Journal of the American Heart Association

ORIGINAL RESEARCH

Anthocyanins Activate Membrane Estrogen Receptors With Nanomolar Potencies to Elicit a Nongenomic Vascular Response Via NO Production

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BACKGROUND: The vascular pharmacodynamics of anthocyanins is only partially understood. To examine whether the anthocyanin-induced vasorelaxation is related to membrane estrogen receptor activity, the role of ERα or GPER antagonism was ascertained on anthocyanins or 17-β estradiol-(E2) induced vasodilatations and NO production.

METHODS AND RESULTS: The rat arterial mesenteric bed was perfused with either anthocyanins or corresponding 3-O-glycosides, or E2, to examine rapid concentration-dependent vasorelaxations. The luminally accessible fraction of NO in mesenteric perfusates before and after anthocyanins or E2 administration was quantified. Likewise, NO-DAF signal detected NO production in primary endothelial cells cultures incubated with anthocyanins or E2 in the absence and presence of ERα (ICI 182,780) or GPER (G-36) selective antagonists. Anthocyanins or corresponding glycosides elicited, within minutes, vasodilation with nanomolar potencies; half maximal anthocyanin response reached 50% to 60% efficacy, in contrast to acetylcholine. The vasorelaxation is of rapid onset and exclusively endothelium-dependent; NOS inhibition annulled the vasorelaxation. The delphinidin vascular response was not modified by 100 nmol/L atropine but significantly attenuated by joint application of ICI plus G-36 (52±4.6 versus 8.5±1.5%), revealing the role of membrane estrogen receptors. Moreover, the anthocyanin or E2-induced NO production was antagonized up to 70% by these antagonists. NO-DAF signal elicited by anthocyanins was annulled by NOS inhibition or by ICI plus G-36 addition.

CONCLUSIONS: The biomedical effect of anthocyanins or 3-O-glycosylates derivatives contained in naturally purple-colored foods or berries is due to increased NO production, and not to the phytochemical's antioxidant potential, highlighting the nutraceutical role of natural products in cardiovascular diseases.

Key Words: anthocyanins ■ endothelium ■ GPER and ERα ■ NO production ■ nongenomic steroidal actions ■ vasodilation

Anthocyanins are water-soluble colored polyphenol pigments synthesized by red, blueish, or darker berries and vegetables.[1,2] Chemically, these compounds belong to a subclass of flavonoids, based on a 3-ring structure; ring C has a charged oxygen heterocycle that at physiological pH loses charge.[1,3,4] Anthocyanins are characterized by the presence of several hydroxyl or methoxylated groups in ring B (Figure 1A), hence the attractive color of berries due to varying proportions of these phytochemicals. Natural anthocyanins are O-glycosylated with either glucose, galactose or disaccharides such as rutinose in the A or C ring, mainly at C3, C5, or C7,[2–5] to distinguish the respective aglycones lacking sugar conjugation. In addition to strong and well-known antioxidant properties,[1,5,6] biological activity of these molecules are emerging such as anti-inflammatory, antidiabetes mellitus, anti-obesity, and cardiovascular effects.[5,7–10] However, these biomedical actions have been examined mainly for aglycones such as cyanidin

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Supplementary Material for this article is available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.119.020498

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JAHA is available at: www.ahajournals.org/journal/jaha

CLINICAL PERSPECTIVE

What Is New?

- Fruit anthocyanins such as delphinidin or structurally related compounds, whether glycosylated or not, are membrane estrogen receptor ligands with a NO-dependent vasodilator effect; this vascular response is nongenomic and unrelated to the anthocyanin's antioxidant capacity.

What Are the Clinical implications?

- Diets rich in berries provide bioactive compounds with proven nutraceutical properties highlight the beneficial role in cardiovascular health care.

Nonstandard Abbreviations and Acronyms

ACh	acetylcholine
D	delphinidin
D3G	delphinidin 3-glucoside
DAF	4 amino 5methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM)
E2	17-β-estradiol
ERα	estrogen receptor alpha
GPER	G protein-coupled estrogen receptor
ICI	ICI 182,780 or fulvestrant (7α,17β)-7-[9- [(4,4,5,5,5 Pentafluoropentyl) sulfinyl]nonyl] estra-1,3,5(10)-triene-3,17-diol
L-NAME	Nω-nitro-L-arginine methyl ester
L-NNA	Nω-nitro-L-arginine
NA	noradrenaline
NO-DAF	signal, nitric oxide fluorescent detection

or delphinidin; it remains uncertain whether conjugated derivatives have similar pharmacological effects.[9] Considering that diet products have 2.5 times more of the 3-O-glucoside anthocyanins than aglycones or 3,5-O- diglycosides,[1] this study aimed at comparing the effect of aglycones with conjugated phytochemicals. As an example of the latter, delphinidin 3-glucoside (D3G), an abundant calafate berry anthocyanin,[11] resulted as potent a vasodilator as delphinidin or acetylcholine, and 100-fold more potent than structurally flavonoids such as quercetin or conjugated derivatives.[11]

Since anthocyanins are structurally related to phytoestrogens such as genistein,[3,12,13] it has not escaped our attention that based on this assumption anthocyanins might have rapid vasodilatory effects comparable to estrogen. Epidemiological studies report that 17β-estradiol (E2) has a cardioprotective effect in premenopausal women linked to the arterial health.[14–17] E2 is known to cause rapid vasodilatation of vascular territories; 2 receptor-mediated mechanisms have been advanced to account for the nongenomic action of E2-induced vasodilatation,[18,19] in addition to its well-documented nuclear steroid action.[17,18,20–22] Over the past decade, several studies illustrate that estrogen binds and activates membrane-bound estrogen receptors (ERα) that signal intracellularly, resulting in endothelial NO synthesis through eNOS activation, causing a subsequent NO production burst.[17,22,23] GPER (G protein-coupled estrogen receptor), is a second membrane-bound estrogen receptor found in endothelial cells, smooth muscle, adipose, and cardiac tissue,[14,19,24,25] that also elicits rapid/nongenomic cardiovascular effects, via a NO-dependent mechanism.[14,15,25,26]

Based in these premises and considering structural similarities between anthocyanins and isoflavones and/or E2, we proposed, as a working hypothesis, that anthocyanins, including 3-O-conjugated glucosides, elicit vasorelaxant responses due to rapid, nongenomic, vascular responses through ERα or GPER activation linked to endothelial NO production. This communication summarizes the use of ERα and GPER pharmacological antagonists to evaluate the vascular responses and NO production elicited by E2, anthocyanins, and structurally related phytochemicals.

METHODS

All data and supporting materials have been provided with the published article. The authors declare that all supporting data are available within the article (and its online supplementary file). Moreover, authors will be available to provide additional information if requested.

Animal Sources and Ethical Issues

One hundred twenty-six male Sprague Dawley rats (180–250 g) were used; animals were bred at the Animal Reproduction Facility of the P. Catholic University and brought to the Animal Housing Facility at the University of Santiago de Chile. Rats were kept in the local facility until use, fed ad libitum purine chow and water; 12 hour light cycles, humidity and temperature were kept constant. Rat handling, animal care, and welfare followed NIH (USA) standard guidelines approval. The Universidad de Santiago de Chile Ethical Committee for the use of animals in biological research approved the specific protocols (776-2017) and supervised our strict adherence to the subscribed guidelines through the Faculty Ethical Committee. Every possible effort to lessen the number of euthanized animals was mandatory, based on the 3Rs ethical principle. Most of the experiments were designed and performed with 4 to

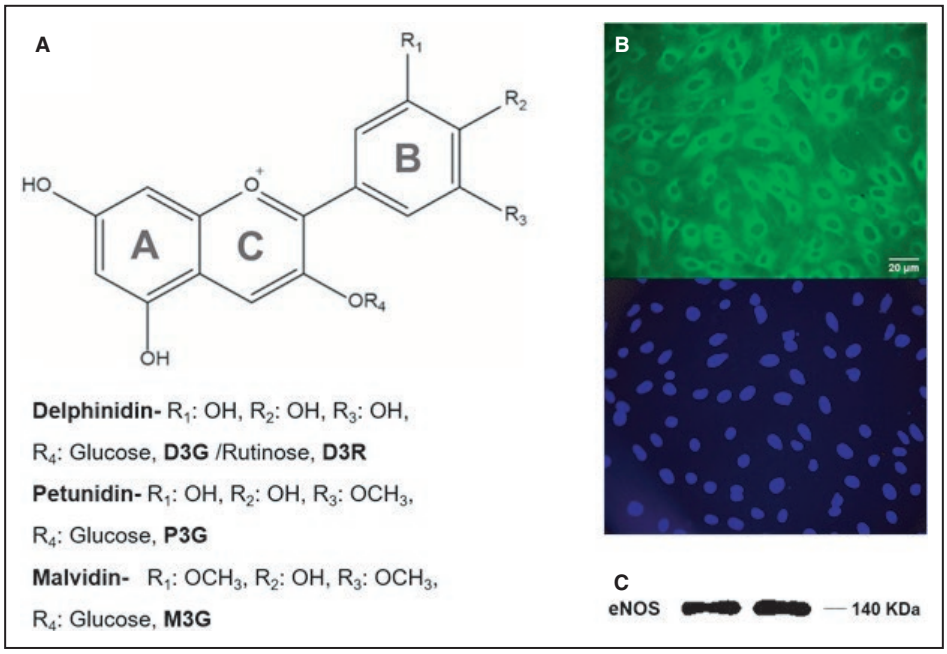

Figure 1. Anthocyanin structures and immune characterization of cultured endothelial cells.
A, Chemical structures of the main anthocyanins examined. **B**, von Willebrand factor immunofluorescence and the corresponding 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) nuclear staining. **C**, Immunoblot analysis of endothelial nitric oxide synthase (eNOS) expression in protein samples extracted from the endothelial cells.

5 individuals; where the statistical significance was evident, the number of rats used was purposely reduced. In these few cases, the number of animals was 3.

Vascular Reactivity Assays and Quantification of Vasodilator Responses; Perfusion of the Rat Arterial Mesenteric Bed

Rats were anesthetized by an i.p. injection of a mixture of ketamine (75 mg/kg) plus xylazine (5 mg/kg). Once anesthetized, a mid-line abdominal incision was performed; the superior mesenteric artery was cannulated with polyethylene tubing and perfused with Tyrode solution (mmol/L: 118 NaCl, 5.4 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 23.8 $NaHCO_3$ and 11.1 D-glucose). Buffer solution was gassed with 95% O_2/5% CO_2 at 37°C and perfused at a flow of 2 mL/min. A pressure transducer was connected at the entrance of the superior mesenteric artery and connected to a Grass polygraph recorder to continually monitor perfusion pressure changes elicited by the anthocyanins and other compounds examined. The mesentery preparations were equilibrated for 20 minutes before starting experiments by perfusing buffer solution in the absence of vasoactive agents; pressure fluctuations were interpreted as changes in the resistance of the arterial mesenteric network. To record and analyze vasodilation, the arterial bed was precontracted with 50 µmol/L noradrenaline (NA), a procedure that increased the perfusion pressure by 30 to 45 mm Hg, a condition that favors vasodilator responses reproducibility.

To assess vasodilator response, the vasculature was precontracted with noradrenaline dissolved in Tyrode buffer; once the increase in perfusion pressure plateaued, several anthocyanin concentrations were perfused in buffer added with NA (see Figure 2 tracing). Ten mmol/L stocks of glycosylated anthocyanins and structurally related compounds were prepared in distilled water and diluted into the Tyrode perfusion solution. In the case of the aglycones, a 10 mmol/L stock solution was prepared in acidified methanol and diluted into the Tyrode buffer. Once the vasodilator response peaked, the mesentery was perfused with regular Tyrode until a full vascular bed relaxation was achieved. Concentration-response protocols were usually performed by examining at least 4 different concentrations of anthocyanins and other flavonoids, in 4 to 6 separate animals. All protocols started with the 50 µmol/L NA-induced contraction followed by perfusion with 1 µmol/L acetylcholine (ACh); in case the ACh-induced vasodilatation did not reach over 80% relaxation, the tissues were discarded likely due to tissue damage during surgery or perfusion; this occurred in 5/100 preparations. See schematic protocol outlined in Figure S1A.

Vasodilatations were analyzed as the percentage reduction of the 50 µmol/L NA-induced contraction

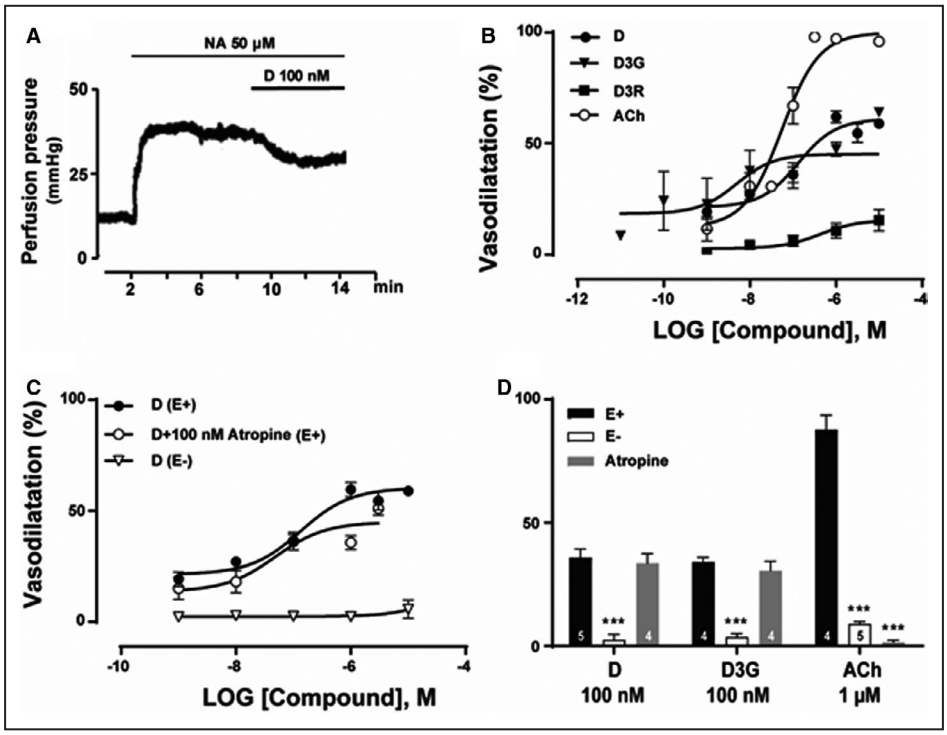

Figure 2. Delphinidin as aglycone or 3-O-glucoside (D3G) elicit endothelium-dependent vascular responses comparable in potency with acetylcholine.
A, Representative experimental tracing shows that perfusion of 100 nmol/L delphinidin (D) induced vasodilatation in the rat arterial mesenteric bed precontracted with noradrenaline (NA). **B**, Mesentery perfusion with delphinidin (D, n=6), delphinidin 3-O-glucoside (D3G, n=6) or delphinidin 3-O-rutinoside (D3R, n=4) concentration-response curves compared with acetylcholine (ACh, n=5). **C**, Delphinidin vasodilator concentration-response curves prior to (n=5) and after mesentery perfusion with 100 nmol/L Atropine (n=3) and in denuded endothelial preparations (E–, n=4). **D**, Comparison of the vasodilatation effect elicited by 100 nmol/L delphinidin (D) or delphinidin 3-O-glucoside (D3G) and 1 µmol/L acetylcholine (ACh) in intact endothelium (E+) or endothelial denuded mesenteries (E–, n=4–5). 100 nmol/L Atropine was perfused 10 minutes before agonist applications in intact endothelium mesenteries. Symbols represent mean values; bars, SEM. Number in columns represent the experiment replication. ***P<0.001.

elicited by 1 nmol/L to 10 µmol/L of flavonoids or E2 standards perfused. Results compared the magnitude of the 1 µmol/L ACh vasodilation in each mesentery preparation. Protocols were replicated in at least 4 separate rats to determinate concentration-response curves for each ligand examined. Following ethical rules to decrease animal euthanasia, on some occasions, a same rat was used to assess more than one anthocyanin or flavonoid. Concentration-response curves were adjusted to sigmoid curves using Graph Pad Prism 8; potency (EC_{50}) and the maximal pharmacological efficacy (E_{max}) was derived from each bioassay. Separate bioassays evaluated E2 or G-1 activity before and after estrogen receptor antagonists.

Denudation of the Mesentery Endothelial Cell Layer

To evaluate the role of the endothelium in the vascular responses elicited by anthocyanins, mesenteries were perfused with 0.1% saponin for 90 seconds to remove a substantial portion of the endothelial layer without damaging the adjacent vascular smooth muscle layers; this procedure was previously reported by Calfio and Huidobro-Toro.[11]

Nonmuscarinic Nature of Anthocyanin-Mediated Vasodilation

To discard the influence of muscarinic receptors in the delphinidin-induced vasodilatation, a concentration-response protocol was perfused with several concentrations of delphinidin before and after a 10 minutes perfusion with 100 nmol/L atropine; the same mesenteries were also examined with several ACh concentrations. In addition, other anthocyanins were also examined in the same experimental condition; in view of the lack of atropine antagonism, this experiment was replicated only 3 times (n=3).

NOS Blockade by a Structural L-Arginine Analog Reduced the Anthocyanins and E2-Induced Vasodilation

The participation of NOS was evaluated in mesenteries before and after 1 hour of tissue incubation with 150 µmol/L Nω-nitro-L-arginine (L-NNA) prior to perfusion with 100 nmol/L of glycosylated or aglycones anthocyanin and structural analogs. The vasodilator response of anthocyanins, other flavonoids, and E2 was evaluated following the same protocol. See schematic protocol outline in Figure S1B. In all cases, mesentery perfusate was collected to examine NO production by anthocyanins and other ligands in control and L-NNA treated tissues. In view of the consistency of L-NNA blockade-induced blunting of NO production elicited by delphinidin or D3G, these protocols were only examined in 4 separate animals to reduce unnecessary animals euthanized. The same applies for the other glycosylated anthocyanins shown in Table.

NO Determinations in the Bioassay Perfusates

The NO released to the perfusate by anthocyanins either glycosylated or as the aglycones as well as other compounds was determined in noncontracted endothelium intact mesenteries, to avoid interference due to NO release triggered by NA vasoconstriction.[27] To determine the luminally accessible NO released to the media, perfusate samples were collected in prechilled 4 mL test tubes every 30 seconds for 2 minutes before, during the 6 minutes anthocyanins perfusion, and for additional 5 minutes after either delphinidin or D3G perfusion, to examine whether NO basal levels recovered upon discontinuing the anthocyanin perfusion (n=7). A similar protocol was designed to examine whether anthocyanin-induced NO release in mesenteries pretreated with 150 µmol/L L-NNA (n=6). See schematic protocol shown in Figure S2.

Endothelial Cell Harvesting for Primary Cultures

The protocol described by Ashley et al,[28] with slight modifications detailed by Donoso et al,[29] was strictly followed. After rat anesthesia, the abdomen was excised at the midline, the superior mesenteric artery cannulated to start mesentery Tyrode buffer perfusion supplemented with penicillin (200 U/mL), streptomycin (0.2 mg/mL), and amphotericin-B (0.5 µg/mL). Few minutes later, the vascular bed was carefully dissected from the live rat, the pancreas was removed and discarded; the mesenteric bed was placed in a Falcon tube and incubated with 5 mL of buffer supplemented with antibiotics plus 0.1% bovine serum albumin (BSA) plus 2 mg/mL collagenase I. Pneumothorax under deep anesthesia and subsequent bleeding was used for rat euthanasia. The tissue was incubated at 37°C for an hour under mild stirring; thereafter, the tissue was centrifuged at 1157 *g* at 4°C for 10 minutes to remove mesentery fat and gross debris. The cell pellet was dissolved in M-199 media plus antibiotics and centrifuged at 289 *g* at 4°C for 5 minutes; the new pellet was resuspended in 36 mL of 199 media plus antibiotics and 20% fetal bovine serum plus 20 µg/mL endothelial cell growth factor (ECGF) supplements. Endothelial cells were seeded in two 24 multi-well plates and incubated at 37°C in a cell incubator with controlled CO_2 pressure until reaching 80% confluence within 3 days. These cells evidenced von Willebrand factor and eNOS expression (Figure 1B, 1C and Figure S4) based on immunoreactive detection. In addition, these cells displayed its characteristic elongated and flattened microscopic morphology.

NO Determinations and Experimental Protocols

Chemiluminescence Assay

This technique determined the NO equivalents present in the perfusate. Protocols assessed whether

Table. Vasodilatation and NO Production Responses Induced by Anthocyanins 3-O-Glucosides Perfused to the Rat Arterial Mesenteric Bed Before and After Nitric Oxide Synthase (NOS) Inhibition

Agonist 100 nmol/L	Vasodilatation (%)		Total NO Released [nmol/L]	
	Control	L-NNA	Control	L-NNA
Delphinidin	36.5±3.2 (5)	1.0±0.2 (4)†	1469±243.8 (7)	397.9±79.3 (6)*
D3G	35.5±5.7 (6)	0.5±0.2 (4)†	1477±329.9 (7)	302.9±68.2 (6)*
P3G	39.1±2.9 (6)	0.7±0.1 (4)†	1449±154.1 (6)	280.7±40.7 (5)†
M3G	38.7±3.5 (4)	1.3±0.9 (4)†	1232±91.8 (6)	304.9±63.3 (5)†
ACh	63.6±7.2 (5)	20.4±7.7 (4)*	1428±281.1 (6)	360.4±87.9 (5)*

The number of animals examined per anthocyanins and acetylcholine (ACh) is indicated in parenthesis. D3G indicates delphinidin 3-O glucoside; L-NNA, Nω-nitro-L-arginine; M3G, malvidin 3-O glucoside; and P3G, petunidin 3-O glucoside.

*P<0.01 and †P<0.001, as compared to controls without inhibitor.

anthocyanins, acetylcholine, E2, G-1 (GPER agonist), ICI (ERα/β antagonist), or G-36 (GPER antagonist) were mesenteric bed perfused to examine NO production; 13 minutes thereafter. NO was quantified by chemiluminescence using a Sievers 280 NOA analyzer (GE Analytical Instruments, Boulder, CO) as previously detailed by Calfío and Huidobro-Toro.[11] In brief, perfusate samples were injected into the reaction chamber of the NOA equipment which contained 100 mg of potassium iodine in 8 mL of glacial acetic acid bubbled with nitrogen used as carrier of the NO produced. This reaction reduced the nitrites in the perfusate sample aliquots to NO, which emitted chemiluminescence upon equipment ozone generation. The response was proportional to the media nitrite equivalents. Equipment calibration was routinely performed using sodium nitrite standards (10 nmol/L to 10 μmol/L). Experimental results were expressed either as the time course of NO equivalents (nmol/L) or as the total NO released above basal values (nmol/L). The area under the curve calculated the NO peak released by the studied ligands.

A separate series of experiments aimed at determining the luminally accessible NO elicited by delphinidin and related anthocyanin agonists or E2 in the presence and absence of estrogen receptor antagonists; to this aim, mesentery perfusates were collected before and after perfusion with the 2 estrogen receptor antagonists (G-36 plus ICI 182,780, n=6, Figure S2); to reduce unnecessary animal euthanasia considering the consistency of these results. Appropriate drug controls were routinely performed.

DAF Fluorophore Studies

This technique determines intracellular NO production by means of a chemical reaction between 4 amino 5 methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate, DAF) and the intracellularly produced NO.[30,31] Cultures of endothelial cells grown in 25 mm diameter cover slips were loaded with 5 μmol/L DAF following a 30 minutes incubation at 37°C, light protected, a time allowing DAF cell absorption and intracellular deacetylation to yield the active fluorophore. 30 minutes after DAF application, cells were rinsed twice with 600 μL of Tyrode HEPES buffer and rested for 15 minutes at 37°C prior to ligand addition; this incubation period allowed completion of fluorophore deacetylation and minimized cell movement elicited by rinsing. Immediately thereafter, cell wells were placed under a Zeiss confocal model LSM 800 provided with a metal device to keep cells at 37°C. The microscope detected fluorescent cells using a 488 Laser with a 40-fold amplification; 633 ms frames were recorded every 15 seconds for a total of 15 minutes. Fluorescence analysis of single cells was determined later using the WCIF image J program, for time course quantification analysis. Similar DAF fluorophore protocols determined intracellular NO in cells pretreated with L-NAME (a well-characterized NOS inhibitor) or ICI plus G-36 after anthocyanin or E2 applications. Parallel control experiments were performed in cells without external chemicals added or with vehicle/solvent applications using the same volume as used with anthocyanins or E2. In addition, the effect of DAF alone was also controlled. See schematic outline of these protocols in Figure S3.

Results compared the NO-induced fluorescence evoked by the application of a single concentration of these compounds. Most of the protocols were performed using endothelial cells harvested from 3 separate rats; the number of cells examined per experiment were 8 to 12. The notation expressed number of rat and cells evaluated (number of rats, total cells examined). Only in few protocols, less animals were used; in such cases, results are shown as preliminary findings.

Chemicals and Drug Providers

Anthocyanins as aglycones or the 3-O-glycosydes were purchased from Extrasynthese (Genay, France) and Sigma-Aldrich (St Louis, MO). Acetylcholine, atropine sulfate, 17 β-estradiol (E2), ICI 182,780, (also known as fulvestrant, referred in the text as ICI), quercetin, and related compounds, L-NNA and L-NAME as hydrochlorides were also purchased from Sigma-Aldrich (St Louis, MO), while G-1 and G-36 were commercialized from Cayman Chemical Company (Ann Arbor, MI). DAPI was purchased from Thermo Fisher Scientific (MA). DAF-FM diacetate was purchased from Molecular Probes Inc (Eugene, OR). Buffer salts and other chemical reagents were purchased from Merck Chemicals (Darmstadt, Germany). Antibodies: von Willebrand factor was a mouse monoclonal from Santa Cruz Biotechnology (TX), while eNOS was a purified mouse monoclonal from BD Biosciences (CA).

Expression of Results and Statistical Analysis

Vasodilator responses were expressed as percentage of the vasodilation; NO values as nanomolar or total NO released (nmoles L^{-1}). The NO-DAF signal was expressed as the normalized fluorescence ((F1−F0)/F0). Data analysis was performed using GraphPad Prism version 8.0 (San Diego, CA) and

IBM SPSS® Statistics (version 25.0) software programs. The Shapiro-Wilk test was used to confirm the normal distribution of data, which was evaluated in each experiment or collection of identical protocols. Student *t* test or Mann Whitney were used to compare and determinate the statistical difference between groups; significance probability was set at $\alpha<0.05$. One-way ANOVA was used to compare the progress of the agonists-induced time courses of NO production. Two-way ANOVA was used to compare NO production time courses by agonists versus antagonists or its controls. Sample size calculations were made with G*Power software (version 3.1). Based on preliminary studies[11] we estimated differences in expected mean vasodilatation/NO production between the different groups with at least 5 rats per treatment. This number of rats will achieve a significance of 0.05 and a power of 80% for differences between means from different groups.

RESULTS

Anthocyanin-Induced Rapid Nonmuscarinic and Endothelium-Dependent Vasodilator Responses

Mesentery perfusion with delphinidin or its 3-O-glucoside (D3G) conjugate, elicited a rapid and concentration-dependent vasorelaxation (see representative tracings shown in Figure 2A), that reached only 50% to 60% maximal effect. The control ACh-induced vasodilatations attained 99.9±3.2% efficacy (Figure 2B). Moreover, delphinidin 3-O-rutinoside (D3R, a conjugated disaccharide) elicited a markedly attenuated and flat concentration-response curve (Figure 2B). In view of the remarkable anthocyanin's potency, we examined whether muscarinic receptors are related to the vasodilatation. Mesentery perfusion with 100 nmol/L atropine failed to interfere with the delphinidin-induced vascular response (Figure 2C) its EC_{50} value before and after atropine treatment was 182.6 versus 139.8 nmol/L, respectively. In controls, atropine blunted the ACh-evoked relaxation, suggesting a nonmuscarinic mechanism. Control protocols indicated that while the 100 nmol/L delphinidin response averaged 36.3±3.9% vasodilation, after atropine it elicited a vasorelaxation of 35.8±3.5%; in the same preparation, the 1 µmol/L ACh response was blocked by 96% following atropine treatment. Parallel experiments showed that endothelium denudation annulled the delphinidin or D3G elicited vascular dilation ($P<0.001$, Figure 2C and 2D), and flattened the delphinidin concentration response curve, an indication that the anthocyanin vasorelaxation depends on endothelium indemnity.[11] Additionally,100 nmol/L atropine failed to antagonize the 100 nmol/L D3G-induced vasorelaxation (Figure 2D).

The Rapid Vascular Anthocyanin Response Involves NO Production

To comprehend the cellular basis of the anthocyanidin-induced vasodilatation, we next evaluated if endothelial NO is involved in the anthocyanidin-induced vasodilator mechanism. Perfusion of the rat mesentery with either delphinidin or D3G elicited within minutes a surge of NO to the tissue perfusate that decayed after anthocyanin perfusion cessation (Figure 3A). Immediately thereafter, the same mesenteries were perfused with L-NNA, an established NOS inhibitor. This enzyme blocker abrogated the rise in mesentery NO production elicited by the anthocyanins (Figure 3A), a clear indication that the vasodilation is totally dependent on enzymatic NO production, confirming endothelium-dependence. In view that 100 nmol/L of either delphinidin or D3G evidenced a similar profile, we next examined whether the perfusate NO rise was concentration dependent. Results show a linear relation between the concentration of either delphinidin or D3G and the total mesenteric perfusates NO detection (Figure 3B). Moreover, the total NO production is proportional to the mesentery elicited vasodilation (Figure 3C), with a R^2 value of 0.895, confirming the relationship between vasodilatation and NO production. The anthocyanins vascular response was concentration-dependent; interpolation of the corresponding concentration-response protocols established the following order of potencies, standardized as the median effective value: D3G (84.1 nmol/L, n=6), petunidin-3G (P3G, 65.8 nmol/L, n=6), and malvidin-3G (17.7 nmol/L, n=4); the maximal response reached was 54.9%, 55.9%, and 48.7% respectively. Compared with the glycosylated anthocyanins, quercetin, the prototype flavonol vasodilator potency was 11.8 µmol/L attaining 99.4% efficacy (n=4). In the same assays, the ACh potency was 43.4 nmol/L with 99.9% efficacy (n=5); the latter used as an internal control. These results are summarized in Table S1.

These observations were further extended and showed that the vasodilator response elicited by 100 nmol/L either D3G, P3G, M3G, or ACh, were significantly reduced by NOS inhibition. Furthermore, the vasodilatation was paralleled by a substantial luminally accessible NO surge to the perfusate that was statistically sensitive to enzyme inhibition, compatible with endothelium NO-dependence (Table).

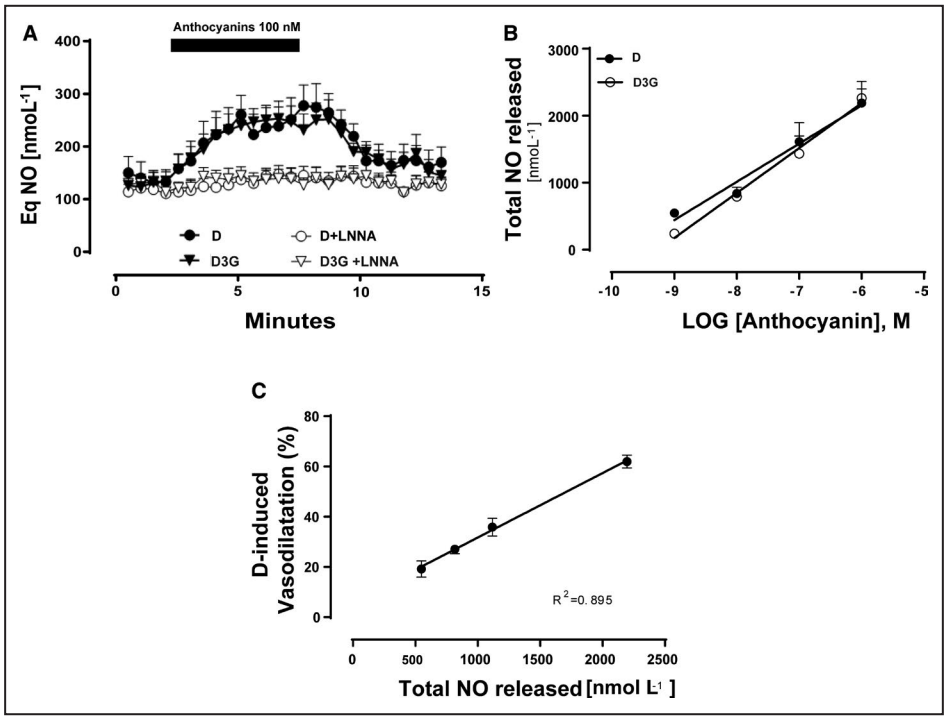

Figure 3. Potent anthocyanin vasodilator effect is NO production associated.
A, Time course of luminally accessible NO elicited following mesentery perfusion with either 100 nmol/L delphinidin (D, black circles, n=7) or delphinidin 3-O-glucoside (D3G, black triangle, n=7) in the rat mesentery vascular bed. Black bar in the top indicates the 6 minutes anthocyanin perfusion before or after 1 hour of nitric oxide synthase (NOS) inhibition using Nω-nitro-L-arginine (L-NNA, open symbols for each anthocyanin, n=6). **B**, Linear relation between the anthocyanin's concentration and the total luminally accessible NO (as before black circles delphinidin [D] and black triangle delphinidin 3-O-glucoside [D3G], n=5 per each anthocyanin). **C**, Linear regression between delphinidin (D)-induced vasodilatation (expressed as %) and the total luminally accessible NO quantified in the mesentery perfusate elicited by each delphinidin (D) concentration; R^2=0.895 (P<0.05, n=4). Symbols indicate the mean values, bars the SEM.

DAF-Fluorescence Revealed Intracellular NO Production by Endothelial Cells

To confirm that the NO detected in mesenteric perfusates is due to endothelial NOS activity, in a next step, we used endothelial cells to assess anthocyanins-induced NO production through the intracellular formation of NO-DAF signal. In these assays control cells fluorescence was maintained constant over 15 minutes (Figure 4A). Application of 100 nmol/L ACh elicited significant NO production; the NO rise was abrogated following L-NAME incubation, a prediction fully supported by the experimental data shown in Figure 4B (P<0.0001). In a control experiment, L-NAME incubation alone did not modify cell fluorescence (Figure 4A). Moreover, 100 nmol/L delphinidin application showed a progressive increase in NO production, annulled by L-NAME cell treatment confirming the enzymatic origin of NO (Figure 4C, P<0.0001 and representative confocal images in Figure S5). Similar findings were likewise observed with 100 nmol/L D3G (Figure 4D, P<0.0001); cell pretreatment with L-NAME abolished the D3G-elicited NO production (Figure 4D, P<0.0001).

Involvement of GPER and ERα in the Fast, Nongenomic, Anthocyanin Vasodilator Response

GPER Ligands

The vasodilator response of anthocyanins was significantly reduced by tissue perfusion with G-36, an alleged pharmacologic GPER antagonist (compare representative tracings in Figure 5A and 5B). The 1 µmol/L delphinidin or D3G-induced vasodilation was significantly attenuated by tissue pretreatment with 1 µmol/L G-36. As a control, GPER agonism elicited by tissue perfusion with either G-1 or E2 mimicked the anthocyanin-induced rapid vasodilatation. While the G-1-induced vascular dilatation was reduced 76.7% by G-36 (P<0.001, Figure 5D), the 3 µmol/L E2-induced vasodilatation was reduced only by 32.04% following G-36 treatment (P<0.05, Figure 5D).

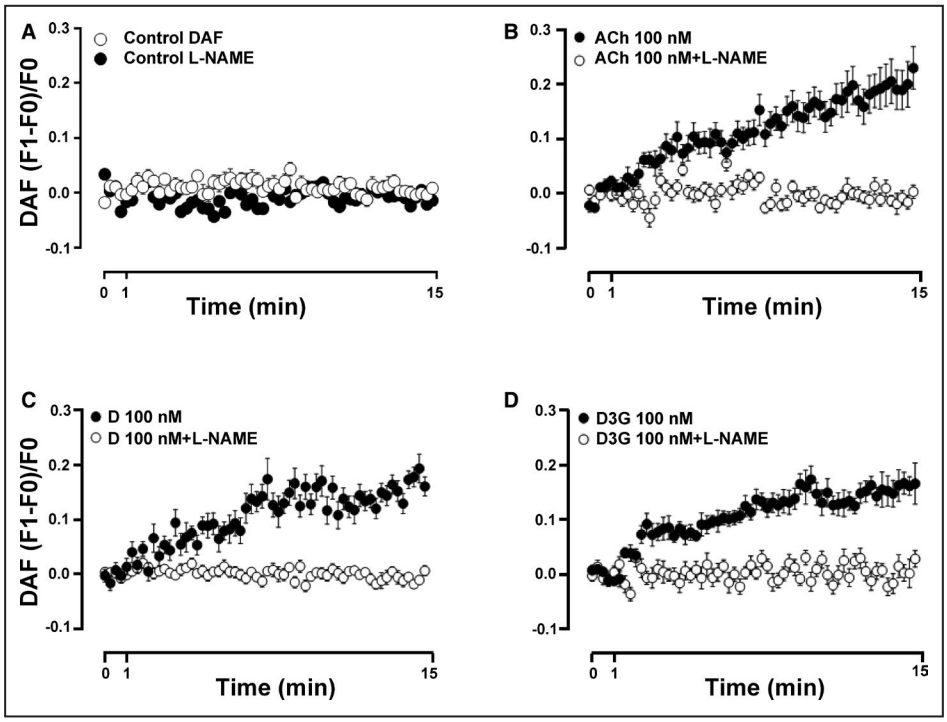

Figure 4. Anthocyanins induced NO production in endothelial cells is annulled by Nω-nitro-L-arginine methyl ester (L-NAME).
NO-DAF signals were determined in single cells using confocal microscopy; primary endothelial cell cultures were loaded for 30 minutes with 5 μmol/L 4 amino 5methylamino-2′,7′-difluorofluorescein diacetate (DAF); 1 minute thereafter, cells were added with 100 nmol/L of agonists. **A**, Control cells loaded with DAF without agonist application (1,11) or separate cells only treated with L-NAME (1,16). **B**, Acetylcholine application (ACh, 3,22) or ACh plus L-NAME (2,26), **C**, Delphinidin (D) application (3,27) or delphinidin (D) plus L-NAME (3,31). **D**, Delphinidin 3-O-glucoside (D3G) addition (3,34 cells) or D3G plus L-NAME (3,28). Symbols indicate the mean value at each time point of fluorescence detection; bars refer to the SEM. After checking for normal curve distribution, 2-way ANOVA showed statistical significance: F values for (**B** through **D**) were F (54, 2484)=8.8; F (35, 1960)=7.2; F (54, 2862)=4.5, respectively (P<0.001); for (**A**) F value was (60, 1525)=1.087 (P=0.30 n.s) for the control assays.

ICI as an ERα Antagonist

While this recognized estrogen receptor antagonist reduced a third of the E2 response (P<0.01, Figure 5E), it failed to antagonize the vasodilatation elicited by 1 μmol/L delphinidin, while in the same tissues it significantly attenuated by 60.23% (P<0.001) the 1 μmol/L D3G-induced vasodilatation (Figure 5E). This is the first experimental finding supporting a difference between the mechanism of delphinidin as its aglycone compared with D3G in the vasodilatation mechanism.

Combined Use of Both GPER and ERα Antagonists

Based on the previous findings, we assessed next whether the joint application of ICI plus G-36 blocked to a larger extent the delphinidin or D3G-induced vasodilatation, implying that both ERα and GPER are involved in the vascular action of the anthocyanins and E2. Present findings fully confirm our proposal demonstrating a substantial and almost complete blockade of the vasodilator response elicited by delphinidin, D3G, or E2 (P<0.001, Figure 5F) in tissues simultaneously preincubated with both antagonists.

Anthocyanins-Elicited NO Production in the Mesenteric Bed or Isolated Endothelial Cells was Abrogated by the Joint Application of GPER Plus ERα Antagonists, Supporting Nongenomic Actions

To further determine whether the anthocyanidins-elicited NO production is antagonized by the simultaneous application of ICI plus G-36, protocols quantified endothelium NO production before and after mesenteries perfusion with these 2 antagonists simultaneously. A representative time course experiment clearly shows that the luminally accessible mesenteric NO production elicited by 1 μmol/L delphinidin was

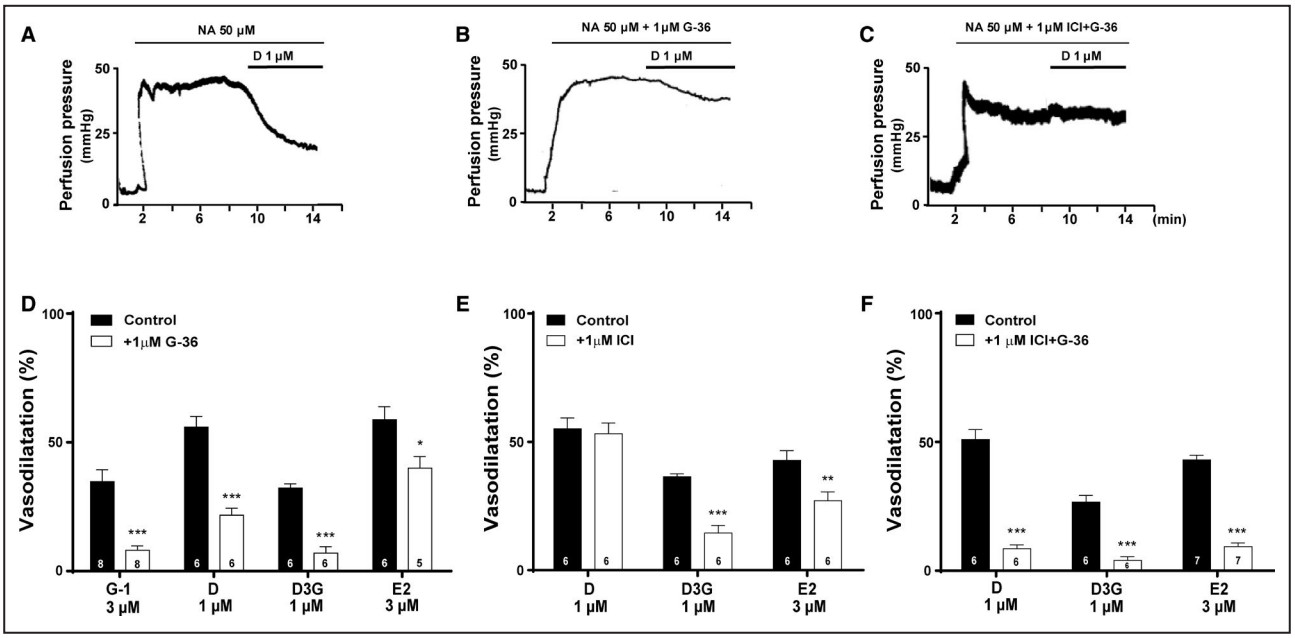

Figure 5. Potent and rapid vasodilator effect of anthocyanins involves estrogen receptor activation.
Representative tracings illustrate the delphinidin-induced vasodilatation in noradrenaline (NA) precontracted rat mesenteries in (**A**) the absence, (**B**) the presence of 1 µmol/L G-36 (a recognized GPER antagonist), and (**C**) the presence of 1 µmol/L G-36 plus 1 µmol/L ICI (2 estrogen receptors antagonists). **D**, Quantification and statistical analysis of the agonist-induced vasodilatation obtained in the absence and in mesenteries preincubated with 1 µmol/L G-36. **E**, Agonist-induced vasodilatation before and after mesentery perfusion with ICI. **F**, Quantification of the vasorelaxation elicited by delphinidin (D), delphinidin 3-O glucoside (D3G) and estradiol (E2) in the same tissues before and after perfusion with the two estrogen receptor antagonists (1 µmol/L ICI plus 1 µmol/L G-36). The same mesentery was used to assess the effect of each agonist in the absence and in the presence of the antagonists. G-1 refers to the synthetic GPER agonist, and E2 refers to 17-β-estradiol, used as an internal control for these protocols' series. Symbols indicate the mean value of mesentery vasodilatation; bars refer to the SEM. Numbers inside columns shows the experiment repetitions in separate rats. *, $P<0.05$; **, $P<0.01$ and ***, $P<0.001$. In all cases, the power of the tests was >80%.

abrogated by the dual application of these estrogen receptor antagonists (Figure 6A). Statistical analysis of these protocols illustrates that the joint perfusion with these two antagonists substantially reduced both the anthocyanidin ($P<0.01$) or E2-elicited NO production ($P<0.01$, Figure 6B).

In further confirmation of the previous findings, we next used primary endothelial cell cultures to detail whether blockade of both ERα plus GPER annulled NO production in endothelial cell cultures. Control time course protocols validated the rise in NO-DAF fluorescence signal elicited by either 100 nmol/L E2, delphinidin or D3G. Additional controls showed that the NO-DAF signal was maintained constant for 15 minutes; moreover, the joint application of 2 antagonists in the absence of ligands, did not modify the cell fluorescence along 15 minutes (Figure 6C). Representative experiments demonstrated the progressive rise in fluorescence following E2, D or D3G additions. Consistent with the results shown in Figure 5, the joint application of ICI plus G-36 abrogated in all 3 cases the NO-DAF signal (Figure 6D through 6F), an indication that both estrogen receptor antagonists are required to annul the NO production elicited by E2 or the anthocyanins. These results provide strong pharmacological support that estrogen receptor activity is mandatory for the nongenomic anthocyanidins-induced vasodilatation, suggesting that anthocyanidins are putative estrogen receptor ligands.

DISCUSSION

Six anthocyanins are mainly present in different proportions in the skin of multiple berries, accounting, in part, for their attractive color variants.[2,3,5] We became interested in studying the vascular properties of these phytochemicals in view of the direct correlation between anthocyanin intake and cardiovascular protective effects.[32] To this aim, we examined the vascular relaxation induced by delphinidin, malvidin, and petunidin and corresponding 3-O-glycosylated derivatives (see Figure 1), that differ in O-methylation of ring B, but not substantially in their nanomolar potency as vasodilators. Based on the lack of atropine antagonism and the markedly reduced antioxidant profile of the key glycosylated anthocyanins, the role of muscarinic receptors as well as the antioxidant potential were discarded in the anthocyanin's mechanism of action in agreement

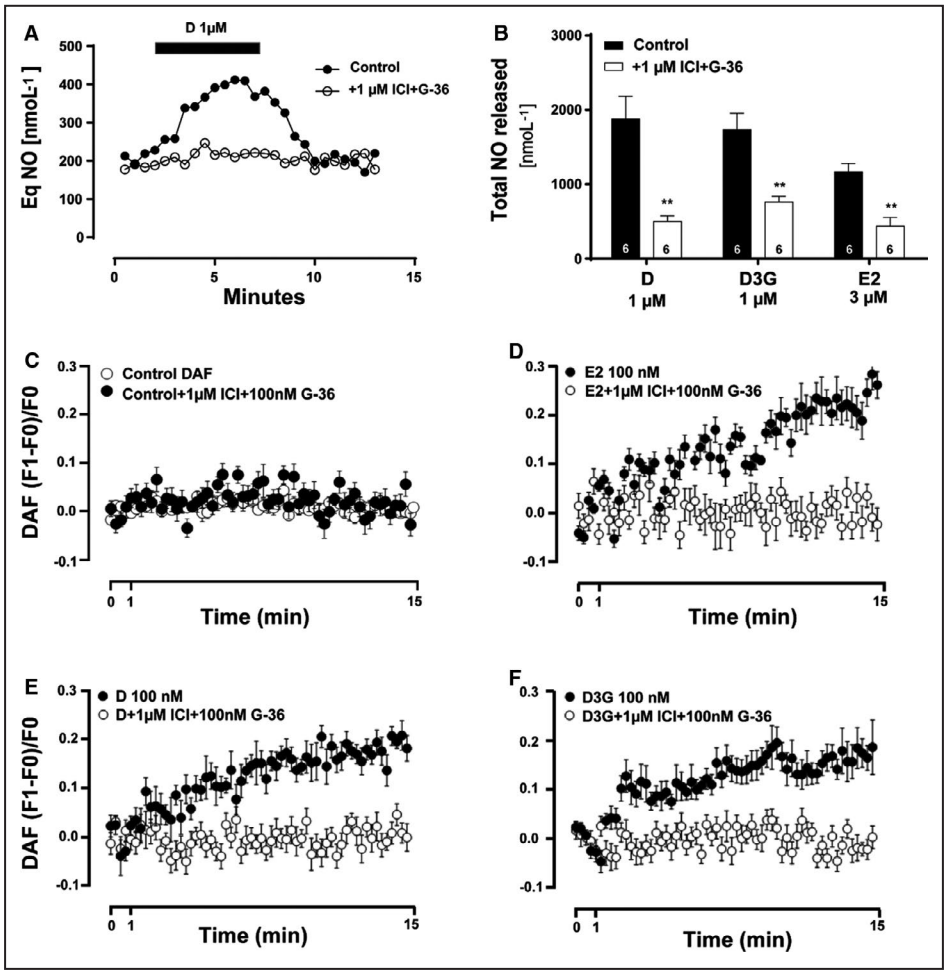

Figure 6. Joint addition of G protein-coupled estrogen receptor (GPER) plus estrogen receptor alpha (Erα) antagonists reduced the anthocyanin-induced NO production in perfused mesenteries or endothelial cells.

A, Representative time course experiment showing the luminally accessible mesentery NO production elicited by 1 μmol/L delphinidin (D) before (control, black circles) and after perfusion with the 2 estrogen receptor antagonists (open circles). **B**, Determination of the luminally accessible NO produced by delphinidin (D), delphinidin 3-O-glucoside (D3G) or estradiol (E2) used as agonists in the absence (black columns) or following the joint perfusion with both antagonists ICI plus G-36 (white columns); **, $P<0.01$. For these series of protocols, the tissue was not precontracted with noradrenaline (NA) to avoid the NO production elicited by the NA contractile response. **C** through **F**, Cultured endothelial cells NO-DAF signals. **C**, Control cells loaded with 4-amino-5-methylamino-2′,7′-difluorescein diacetate (DAF) without agonist application or separate cells treated only with ICI plus G-36. Representative time course protocols show that joint application of ICI plus G-36 annulled NO-DAF signal in E2 [**D** (1,9)], in delphinidin [**E** (1,12)] or in D3G [**F** (1,16)] treated cells. Symbols indicate the mean values; bars, the SEM. Number of cells studied per experimental condition is indicated in parenthesis for each protocol. In all experimental cases shown, the test power was >75%. G-36 refers to the synthetic GPER antagonist and ICI refers to 2 estrogen receptor antagonists.

with a previous group report.[11] Considering that the rapid vascular response of anthocyanins is annulled by NOS inhibitors, and the vascular response is dependent on endothelium indemnity,[11] we infer that NO is the main intracellular mediator of the anthocyanin-induced vascular response and further conclude that anthocyanins act as NO production modulators, rather than oxidizing scavengers. Two separate methods were used to evaluate NO production. The chemiluminescence assay examined the luminally accessible NO to the mesenteric effluent[11,27,33] while the NO-DAF signal allowed the detection of intracellular NO production by endothelial cells.[30,31] Both methods consistently demonstrated a rapid burst of NO availability annulled following NOS inhibition; confirming that the anthocyanins-induced NO surge is of enzymatic origin

and produced by endothelial cells, pointing to NOS as a main direct or indirect bioactive molecules target.

In contrast with the anthocyanins, structurally related flavonoids such as quercetin only partially depend on NO production[34] (C. Calfío, PhD, unpublished data, 2020), suggesting that flavonoids might have more than one target to elicit vasorelaxation. This finding supports stringent anthocyanins structural requisites, a result compatible with the proposal that anthocyanins might act as membrane estrogen receptor ligands. The fact that delphinidin 3-O-derivate is almost equipotent to its aglycone as a vasodilator remains puzzling, considering its polar and bulky sugar moiety a finding reflected by its log D partition coefficient at pH 7, which is 1.35 for delphinidin as compared with −0.78 for D3G. Several possibilities may be discussed, including D3G tissue metabolism to the aglycone, preferential intracellular uptake through GLUT transporters or other proteins, bilitranslocase-mediated membrane transport might also contribute to the trans-membrane passage of anthocyanins into the vascular endothelium to modulate NOS activity.[35–38] In view that the time course of NO production is remarkably similar between delphinidin and its 3-O-derivative we deemed unlikely that D3G must be metabolized in the mesentery to elicit the observed vascular response unless this process is rapid. Moreover, we are aware that UDP-glucose or UDP-galactose are P2Y14R ligands[39] exemplifying that ligand glycosylation does not interfere with P2Y14R activation, much in the same way that current results suggest that anthocyanins, glycosylated or not, may act as ERα or GPER ligands. The strict structural requirements of anthocyanins vascular responses further support the contribution of a receptor(s) in the current proposal. In contrast to rutin (D3R), D3G and delphinidin are equipotent vasodilators. Moreover, quercetin, a structural anthocyanin analog has distinct vascular targets, although it might act as a GPER ligand,[14,40] the vasodilatory action of quercetin is related to a dual mechanism linked to an endothelial mechanism plus a preponderant smooth muscle action.[34,41,42] While the present study was performed in males, subsequent to the Calfio and Huidobro-Toro[11] studies, female and male rats have an equal GPER expression during adulthood with a loss of expression at older ages.[19,25] In this regard, we did not examine female rats, a question that is currently analyzed by others. Notwithstanding, we noted that in old male rats, the vascular responses elicited by delphinidin and D3G were markedly blunted (preliminary result), a finding that could be accounted by the loss of GPER expression with age.[19,25]

Genistein, a natural isoflavone structurally related to anthocyanins, is regarded as a prototype phytoestrogen[13]; delphinidin exerts phytoestrogen effects in MCF-7 cells (breast cancer) to induce cell proliferation[43] and stimulates ERα transcriptional activity in human ERα reporter assays.[44] Based on these premises, we inquired whether the nanopotency of

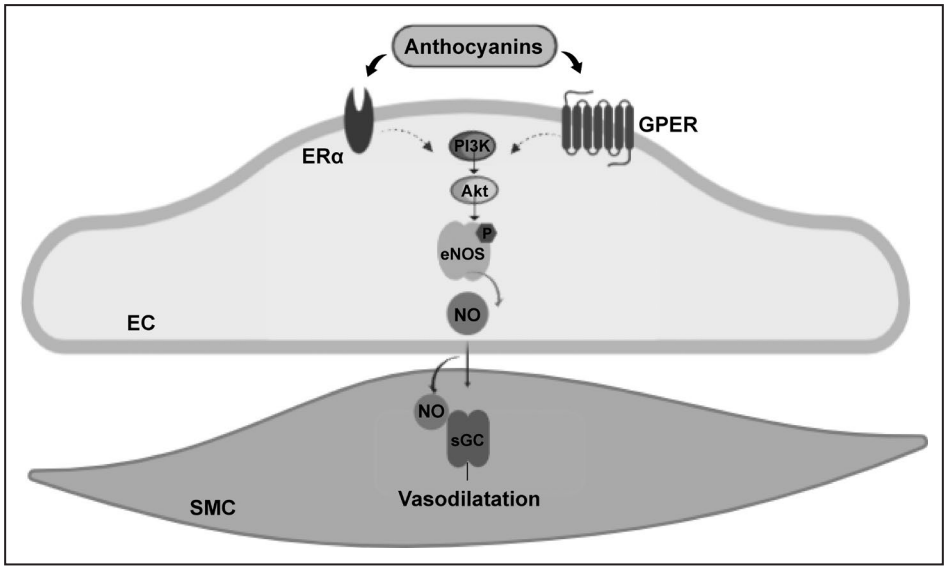

Figure 7. Heuristic model illustrates the anthocyanin-induced rapid vasodilatation.
Nongenomic vascular response of anthocyanins (delphinidin [D] or delphinidin 3-O-glucoside [D3G]) activate GPER or ERα expressed in the plasma membrane endothelial cells. The anthocyanins bind to GPER or ERα leading to protein kinase activation to initiate the eNOS phosphorylation and subsequent NO production increase, ensuing a potent relaxant effect of the adjacent layers of smooth muscle cells. Akt indicates protein kinase B; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; ERα, estrogen receptor alpha; GPER, G-protein estrogen receptor; PI3K, phosphoinositide 3-kinase; sGC, soluble guanylyl cyclase; and SMC, smooth muscle cell.

anthocyanins plus a likely signaling pathway associated to NO-production could be compatible with the proposal that the anthocyanin action is estrogen receptor mediated. In support of our proposal delphinidin might target ERα[45] in the plasma membrane of endothelial cells, mimicking E2 activity, indicating that the anthocyanin-induced vasodilation is likely due to the activation of a fast-nongenomic mechanism like the E2 vascular response. Two estrogen membrane receptors have been described in endothelial cells: ERα and GPER,[14,15,19,25,46] both intimately associated to NO-dependent vasorelaxation. Since pharmacological specific antagonists are available for both types of receptors[14,18,26,47] we challenged the anthocyanin responses in the presence of these antagonists. The present results strongly suggest that both ERα and GPER receptors participate in the anthocyanin-induced vasorelaxation associated to endothelial NO production. To annul the vascular responses of anthocyanins and E2, the joint blockade of these estrogen receptors is mandatory; the NO production elicited by anthocyanins, was consistently reduced by over 70% by the combined exposure of these antagonists. These findings are fully compatible with our proposal that the anthocyanin-elicited vasorelaxation is associated to a rapid, nongenomic ERα and GPER response.

Under the current experimental conditions, the lack of delphinidin antagonism by ICI treatment, at the concentration examined, might be interpreted to indicate that this compound has a relative lower affinity membrane binding site for the ERα compared with GPER. Despite the fact that ICI is an ERα/β antagonist, it has agonist activity in GPER,[26,48] supporting that ICI-treatment would facilitate the GPER modulation by anthocyanins. Although, anthocyanins activate both estrogen receptors and lead to a compounded vasodilatory response based on endothelial NO production; the intracellular mechanisms accounting for the NO production need to be examined in further details, considering that ERα and GPER activation mediate rapid cellular signaling events including Ca^{2+} mobilization, ERK and PI3K/Akt activation.[15,17,19,22,25,49] We are actively pursuing these protocols to highlight the cardiovascular effects of GPER associated to nonestrogenic ligands. Although details of how these receptors interact in the plasma membrane are lacking, 2 findings are relevant. The receptors might be palmitoylated and form dimers and/or interact with caveolin, a finding that favors eNOS activity, since the latter is a caveolin-related enzyme.[22,50] Collectively, the heuristic model illustrated in Figure 7 summarizes present results illustrating the 2 estrogen cell membrane receptors; the signaling pathways of both receptors lead to increase NO production via eNOS Ser^{1177} phosphorylation.

15,17,19,22,25,49

In sum, present results are compatible with the proposal that the rapid vasodilatation elicited by anthocyanins is due to a nongenomic mechanism, related to cell membrane estrogen receptor activity, an effect that does not correlate with the redox potential of these molecules. Moreover, the anthocyanins-induced vasodilatation fully depends on the activation of both membrane-bound estrogen receptors, ERα plus GPER, with NO as a final endogenous bioactive intracellular messenger molecule. As to whether anthocyanins might be considered ligands for these receptors remains to be further investigated but opens exciting perspectives for their use as novel functional foods. Red fruits rich in anthocyanins and red wines, have a nutraceutical value with beneficial health protective properties for both women and men suffering from vascular diseases, closely related to the proposed mechanisms.

ARTICLE INFORMATION

Received December 23, 2020; accepted June 11, 2021.

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Acknowledgments

The authors thank Drs E. Leiva and D. Riquelme for NO-DAF fluorescence signal protocol supervision and D. Valdés for prompt access to confocal microscope scheduling and expert guidance in the instrument use.

Sources of Funding

Calfío was awarded an intramural postdoctoral training grant of Scientific and Technological Research Direction (DICYT) No. 0219143GHT_POSTDOC. The National Fund for Scientific and Technological Development (FONDECYT) 017-0842 provided partial funding; additional financial support was derived from Center for the Development of Nanoscience and Nanotechnology (CEDENNA), grant AFB 180001.

Disclosures

The authors declare no conflict of interest.

Supplementary Material

Tables S1
Figures S1–S5

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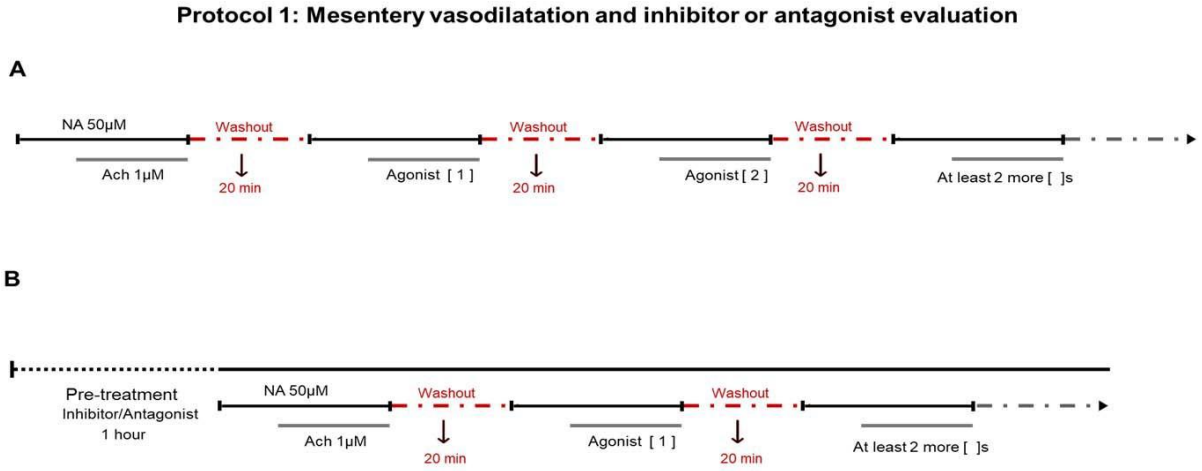
SUPPLEMENTAL MATERIAL

Table S1. **Anthocyanin´s vascular responses.**

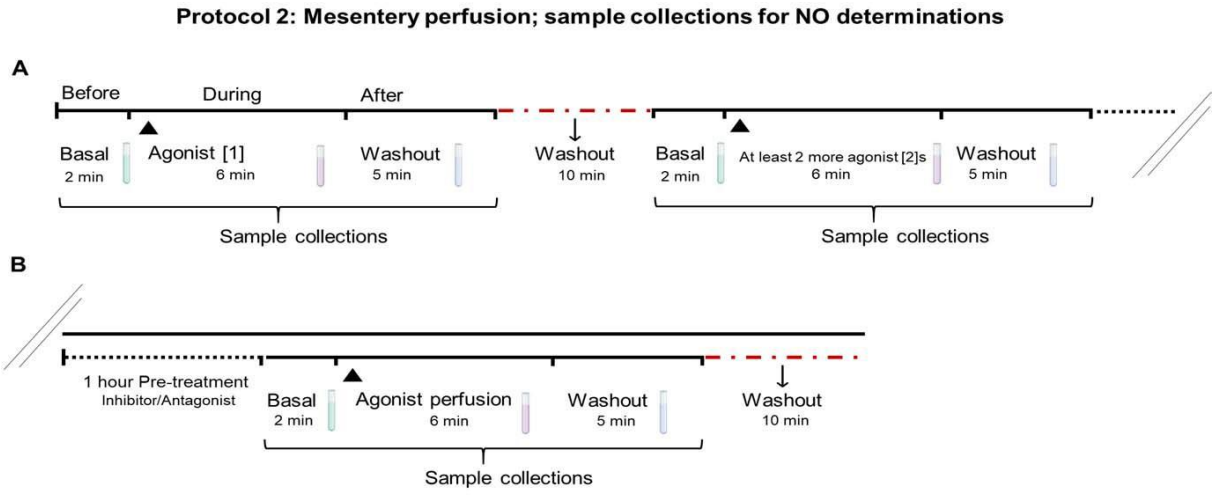
Agonists	-LOG EC50	EC50 [nM]	Emax (%)	n
Anthocyanins				
Delphinidin, **D**	6.83 ± 0.14	182.6	60.3 ± 5.1	5
Delphinidin-3-glucoside, **D3G**	7.56 ± 0.33	84.1	54.9 ± 4.5	6
Delphinidin-3-rutinoside, **D3R**	6.32 ± 0.17	546.5	15.3 ± 4.2	4
Petunidin, **P**	7.25 ± 0.31	87.4	44.1 ± 2.1	3
Petunidin-3-glucoside, **P3G**	7.50 ± 0.35	65.8	55.9 ± 3.7	6
Malvidin, **M**	7.21 ± 0.31	113.5	32.5 ± 4.2	4
Malvidin-3-glucoside, **M3G**	7.76± 0.07	17.7	48.7 ± 4.0	4
Flavonols				
Quercetin, **Q**	4.93 ± 0.06	11864	99.4 ± 4.1	4
Control				
Acetylcholine, **Ach**	7.42 ± 0.13	43.4	99.9 ± 3.2	5

Figure S1. **Schematic representation of the protocols outlined to quantify vasodilation before or after NOS inhibition or the use of receptor antagonists.**



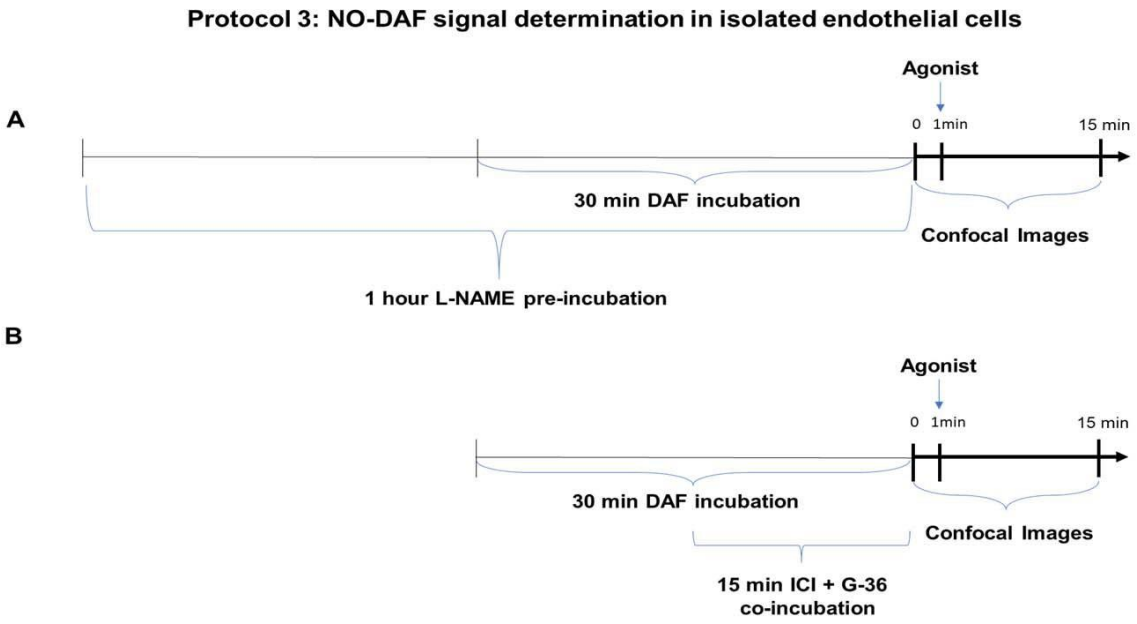
(A) Concentration-response agonist' vasodilatation. (B) Pretreatment with inhibitor or receptor antagonists. Note that four agonists concentrations were routinely used for concentration-response protocols. After each washout period, the mesentery perfused with 50 μM noradrenaline to elicit mesentery vasocontraction.

Figure S2. **Schematic representation of the protocols outlined to collect the mesentery perfusates following agonist applications to determinate NO production.**



In control experiments
(A) and following mesentery pre-treatment with inhibitors or receptor antagonists (B). Note that for these experiments no vasocontriction with noradrenaline was used.

Figure S3. **Schematic representation of the protocols outlined to detect NO-DAF signal from isolated endothelial cells using confocal microscopy.**

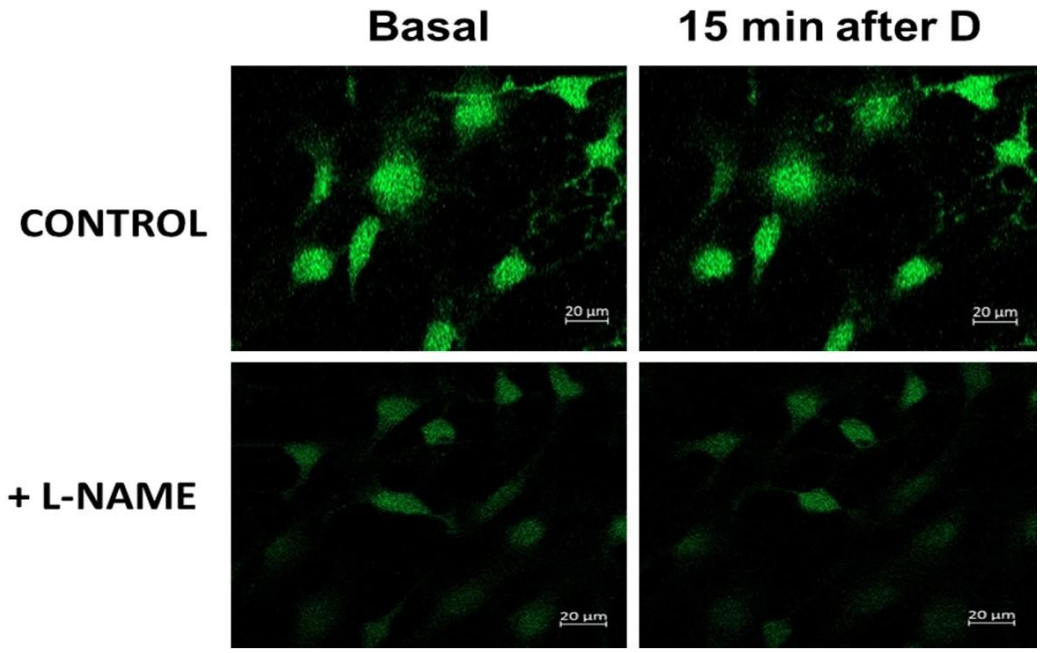


In the experiments where NOS-inhibitor L- NAME was used, cells were preincubated for 1 hour prior to agonist addition (A); the control agonists experiments were performed without L-NAME pre-incubation. (B) Summarized protocols used to assess the effect of the co-incubation of two estrogen receptor antagonists. In all cases, DAF was applied 31 minutes before agonists application.

Figure S4. **eNOS expression in endothelial cells (primary cultures) using eNOS mouse monoclonal antibody.**

Blot shows four lane replicates; molecular weigth markers for 140 kDa.

Figure S5. Representative confocal images of endothelial cells isolated from the mesentery bed.



Upper images illustrates the increase of the NO-DAF signal elicited by 100 nM delphinidin. Lower images show the blockade of the NO-DAF signal in L-NAME pre-treated cells.