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Journal of Cellular Biochemistry 113:1386–1396 (2012)

Journal of Cellular Biochemistry

Procyanidins From Grape Pomace Are Suitable Inhibitors of Human Endothelial NADPH Oxidase

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ABSTRACT

Procyanidins have been associated with a reduced risk of cardiovascular diseases such as atherosclerosis. However, the molecular mechanisms underlying this benefit are not fully understood. Increased reactive oxygen species (ROS) production generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a common problem in different cardiovascular diseases. Our objective was to evaluate the effects of procyanidin-rich fractions from distilled grape pomace on NADPH oxidase activity in human umbilical vein endothelial cells (HUVEC). Three differently polymerized and galloylated procyanidin fractions were analyzed for their NADPH oxidase inhibitory activity in cell lysates and in HUVEC cultures. All of the three fractions, up to 1 μg/ml, equally inhibited isolated NADPH oxidase in HUVEC lysates in a concentration-dependent manner and independently of any superoxide anion scavenging activities. The procyanidin fractions even blocked NADPH oxidase activity in intact HUVEC, inhibiting ROS production at both extra- and intracellular levels. The fractions achieved the same effects that known NADPH oxidase inhibitors, such as diphenylene iodonium and apocynin, but they presented better hydrosolubility. Our results demonstrated that procyanidin from grape pomace inhibit human endothelial NADPH oxidase regardless of their polymerization degree and galloylation percentage. Therefore, procyanidins are suitable NADPH oxidase inhibitors which could serve as models for therapeutic alternatives for cardiovascular diseases. J. Cell. Biochem. 113: 1386–1396, 2012.

KEY WORDS: GRAPE POMACE; HUMAN ENDOTHELIAL CELLS; NADPH OXIDASE; PROCYANIDINS; REACTIVE OXYGEN SPECIES

Procyanidins are polyphenol compounds from the flavonoid group which constitute oligomers or polymers of flavan-3-ols which exclusively consist of catechin and/or epicatechin units. They are widely found in cereals, vegetables, and fruits, such as grapes, berries, and apples, and also cocoa. Procyanidin-rich diets have been associated with a reduced risk of cardiovascular diseases and atherosclerosis [Rasmussen et al., 2005]. However, the molecules responsible and the mechanisms underlying the vascular responses are not fully understood. Grapes, particularly grape seeds and skin, and their derived products, are some of the main dietary sources of this type of product [Gu et al., 2004]. With regard to this, some studies showed different mechanisms by which procyanidins from grapes can contribute to cardiovascular benefits. Among them, hypotriglyceridemic actions in rats [Del Bas et al., 2008], attenuation of in vitro foam cell formation [Terra et al., 2009a], anti-inflammatory systemic modulation in a rat model of low-grade inflammation [Terra et al., 2009b], endothelium-dependent vasorelaxation [Dell'Agli et al., 2004], cardioprotective effects [Bagchi et al., 2003], and in vivo inhibitory effects on platelet aggregation [Vitseva et al., 2005] have been described. Furthermore, the well-documented antioxidant capacity of procyanidins continues to be one of their most promising properties at the cardiovascular level.

Additional supporting information may be found in the online version of this article.

Ezequiel Álvarez and Bruno K. Rodiño-Janeiro contributed equally to this study.

Grant sponsor: *Xunta de Galicia* (Spain); Grant number: Isidro Parga Pondal programme and 2007/PX046; Grant sponsor: Ministry of Science and Technology; Grant number: AGL2006-12210-C03-03.

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Received 25 November 2010; Accepted 22 November 2011 • DOI 10.1002/jcb.24011 • © 2011 Wiley Periodicals, Inc.
Published online 1 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

However, research on procyanidins is limited and many questions still remain to be answered. The main difficulty in studies on procyanidins is probably that of obtaining them in an individual molecular form. The complete purification of a procyanidin with a degree of polymerization (DP) above five monomer units is almost impossible. Therefore, mixtures of different polymerizations are often employed for studying the structures and properties of procyanidins [Guyot et al., 2001]. Moreover, the synergistic effects of active mixtures make plant extracts and fractions more interesting than pure compounds for functional food applications.

The increased production of reactive oxygen species (ROS) has been related to vascular diseases, such as atherosclerosis, hypertension, diabetes vasculopathy, and restenosis [Taniyama and Griendling, 2003]. This increase seems to contribute to endothelial dysfunction, which is an early step of atherosclerosis. At the center of ROS production is the superoxide anion, from which other oxidants, such as hydrogen peroxide, hydroxyl radicals, and peroxynitrite can be derived [Wolin, 2000]. The major source of superoxide anions in the vasculature is reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is a multisubunit enzymatic complex which differs from the phagocytic NADPH oxidase. This membrane enzyme transfers an electron from NAD(P)H to oxygen, rendering the superoxide anion (O_2^-) and NAD(P)$^+$. There are five isoforms of the central membrane-bound subunit of the complex, termed Nox1 to Nox5. Some of these homologs are present in all vascular cells: endothelial cells, smooth muscle cells, fibroblasts, and macrophages [Lassegue and Clempus, 2003]. It is possible that each Nox protein serves a specific biological function and that the different intracellular locations of each Nox protein determine their distinct roles in cell functions [Yokoyama and Inoue, 2004]. Recently, NADPH oxidase expression and superoxide anion production were shown to correlate with the severity of atherosclerosis [Sorescu et al., 2002], plaque stability [Azumi et al., 2002], oxidative stress in coronary artery disease [Guzik et al., 2006], and plasma metalloproteinase-9 levels [Zalba et al., 2007]. These all point to the possibility of NADPH oxidase being a new therapeutic target for atherosclerosis. However, there are no inhibitors of this enzyme that can be used in therapeutics, nor there are any specific inhibitors of each of the isoforms. Therefore, it would be very useful to find new specific and safe inhibitors for this enzyme and its isoforms because of their possible therapeutic utility against vascular diseases [Cai et al., 2003].

The effects of procyanidins on NADPH oxidase activity have been little studied. Recently, grape seed proanthocyanidins from a commercial product showed marked inhibition of NAPDH oxidase activity induced by high glucose in rat vascular smooth muscle cells. The phosphorylation and membrane translocation of Rac1, p47phox, and p67phox subunits leading to NADPH oxidase activation were remarkably disrupted by these proanthocyanidins [Wang et al., 2010]. The inhibitory activity of some flavan-3-ol monomers and their metabolites has been tested by Steffen et al. [2008]. In their study, (−)-epicatechin and (+)-catechin proved to be superoxide scavengers but did not inhibit NADPH oxidase activity, whereas the reverse pattern was observed for the methylated metabolites of epicatechin. The dimer procyanidin B2, the only procyanidin included in the study and (−)-epicatechin glucuronide, an in vivo metabolite of epicatechin, were superoxide scavengers and inhibited NADPH oxidase. Therefore, further investigations are needed to assess the effects of procyanidins on NADPH oxidase activity.

Taking into account all of the above, the following are of special interest for cardiovascular research: (1) to find drugs for new therapeutic targets, such as vascular NADPH oxidase, in particular for NADPH oxidase from the endothelium, and (2) to obtain data about the possible action of procyanidins, one of the most abundant types of flavonoids in the human diet, on the main enzymatic source of superoxides in vascular tissue. Therefore, in the present work, we obtained three different procyanidin-rich fractions from an aqueous extract of grape pomace, a commonly available by-product of the wine industry, and we studied their effects on NADPH oxidase activity in human endothelial cells.

MATERIALS AND METHODS

DRUGS AND MATERIALS

The following drugs were purchased from Sigma–Aldrich (Madrid, Spain): apocynin, 5(6)-carboxy-2′, 7′-dichlorofluorescein diacetate (c-DCFDA), cytochrome c, diphenylene iodonium (DPI), lucigenin, NADH, NADPH, nitroblue tetrazolium (NBT), NP-40, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), plumbagin, phenazine methosulphate (PMS), superoxide dismutase (SOD), and sodium deoxycholate sodium dodecyl sulphate. Except for DPI (20 mM), c-DCFDA (10 mM), and plumbagin (100 mM), dissolved in pure dimethylsulphoxide (DMSO) and then diluted in HEPES buffer solution (HBS) daily for the experiments, all other reagents and F4–F6 fractions were dissolved directly in distilled water or HBS. The final concentration of DMSO never exceeded 0.01% in the experiments and proper controls were always made to assess any effect of the vehicle. The equipment, media, and specific reagents or antibodies employed in the different techniques are indicated in the corresponding sections. All other reagents used in the experiments and in the preparation of work solutions were of the best available quality.

OBTAINING THE GRAPE PROCYANIDIN FRACTIONS

The starting material, provided by Aguardientes de Galicia, S.A. (Vedra, A Coruña, Spain), was the residue left after distillation and pressing of the grape pomace of *Vitis vinifera* (var. Albariño) and consisted of skin, seeds, and a small number of stems. This by-product was acquired in January, immediately after distillation, and was frozen (−80°C) until use. The pomace employed in this study corresponded to 2007, and the seed content was around 40%.

The grape pomace was subjected to aqueous extraction under the selected conditions [Guerrero et al., 2008] to render the crude extracts which were then solvent fractionated, essentially as described by Torres and Bobet [2001], to select the aqueous fraction which contained the larger-sized polyphenols. This aqueous fraction was further sub-fractionated according to molecular size in a Sephadex LH-20 column eluted in a gradient of methanol–water–acetone [Jerez et al., 2007].

To obtain the polyphenolic profile [the mean degree of polymerization (mDP) and the percentage of galloylation (%G)],

the fractions were depolymerized by means of acid thiolysis with cysteamine, as described Torres and Selga [2003], because RP-HPLC showed a broad hump at 280 nm, thus indicating polymerization. After treatment, the terminal flavan-3-ol units were released intact whereas the extension moieties were released as cysteamine derivatives. Two parameters were calculated for each fraction: mDP (total nmol/nmoles terminal units) and %G ($100 \times$ [total galloylated nmols/total nmol]). Three procyanidins fractions named F4, F5, and F6 were selected to study their effects on NADPH oxidase activity.

HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC) CULTURE

This study was conducted according to the guidelines laid down by the Declaration of Helsinki and all procedures involving human subjects were approved by the Galician Ethics Committee of Clinical Investigation (*Comité Ético de Investigación Clínica de Galicia*). Written informed consent was obtained from donor mothers of umbilical cords. The HUVEC were isolated from freshly obtained umbilical cords [Alvarez et al., 2010]. Moderate lumen digestions of the umbilical veins at 37°C with 22 U/ml collagenase IA (Gibco, Invitrogen) in HBS rendered the HUVEC. The isolated cells were seeded onto gelatine pre-coated surfaces and grown to confluence in a humidity saturated 5% CO_2 atmosphere at 37°C with supplemented endothelial cell growth medium (Promocell GmbH) containing 2% fetal calf serum, 0.4% endothelial cell growth supplement, 0.1 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, and 1 ng/ml basic fibroblast factor (all from Promocell GmbH), and an antibiotic mixture of 100 U/ml penicillin and 100 μg/ml streptomycin. A positive characterization of the endothelial cells was made using polyclonal rabbit anti-human von Willebrand factor (Dako) and monoclonal mouse anti-human CD31 (Dako). Cells from 4 to 10 passages were used for the experiments.

CELL VIABILITY EXPERIMENTS

Cell integrity was analyzed by the MTT method. For these experiments, the HUVEC were prepared and placed in 96-well microplates as described above. After serum and supplement deprivation, the HUVEC were incubated for 6 h with different concentrations of F4, F5, or F6 fractions (100 ng/ml–5 μg/ml), apocynin (100 μM), DPI (60 μM), and plumbagin (5 μM), and in the presence of complete medium or a medium deprived of serum and supplements (deprived medium) for the control. After this time, MTT dissolved in culture medium was added to the wells at a final concentration of 0.5 mg/ml and maintained in the incubator for 4 h. Then, MTT formazan crystals were dissolved by adding them to a solution of 0.1 M HCl in 10% (w/v) sodium dodecyl sulphate (SDS) with gentle stirring. The absorbance at a wavelength of 550 nm was measured spectrophotometrically for each well in an ELISA-type plate reader (Multiscan Ex, Thermolabsystems).

NADPH OXIDASE ACTIVITY IN HUVEC LYSATES

In order to measure the direct effects of procyanidin fractions on the isolated NADPH oxidase, the activity of this enzyme was estimated in HUVEC lysates obtained chemically or by sonication, using two different methods. In the first method, NADPH oxidase activity was measured by lucigenin-enhanced (100 μM; Sigma–Aldrich) chemiluminescence, as previously described [Orallo et al., 2002]. Briefly, HUVEC cultures in 75 cm^2 flasks were lysed on ice with 1.5 ml lysis buffer of the following composition: 10% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 150 mM NaCl, 10 mM NaH_2PO_4, 2 mM EDTA, and a protease inhibitor cocktail (P2714; Sigma–Aldrich). The HUVEC lysates were maintained at an ice-cold temperature until the experiments. The NADPH (100 μM)-induced superoxide anion production of the HUVEC lysates (40 μg of protein) was measured during 2 min in a luminometer (BG-250; Optocomp II, MGM Instruments, Inc.) in test solutions with lucigenin (100 μM) made up in HBS of the following composition (mM): 110.0 NaCl, 5.0 KCl, 0.5 NaH_2PO_4, 0.5 KH_2PO_4, 1.5 $CaCl_2$, 2.0 $MgCl_2$, 10.0 $NaHCO_3$, 0.5 EDTA, 10.0 HEPES, and 10.0 glucose (pH 7.4). The F4, F5, and F6 fractions (100 ng/ml–5 μg/ml), DPI and SOD, and appropriate controls were incubated with the lysates for 15 min at room temperature prior to measurement. Specificity for the superoxide anion and NADPH oxidase activities was confirmed by SOD (100 U/ml) or DPI (100 μM), respectively. The data, collected as relative luminescence units, were plotted versus time, and the area under the curve was used for analysis. The results were expressed as a percentage of the area under the curve after subtracting the non-DPI-sensitive percentage of the response.

In the second method, NADPH oxidase activity was measured by following photometrically NADPH consumption using the method described by Steffen et al. [2008]. Briefly, confluent HUVEC were scraped off 75-cm^2 flasks and suspended in a serum-free and supplement-free culture medium. The cells were pelleted by centrifugation (800*g*, 4°C, 5 min), the supernatant was discarded, and the pellets were resuspended in 50 mM phosphate buffer at pH 7.4. Then, the cells were disintegrated by sonication with a probe sonifier for three cycles of 20 s at 0°C.

The sonicate of 1.5×10^6 cells was added to a final volume of 1 ml 50 mM phosphate buffer, pH 7.4, containing varying concentrations of F4, F5, and F6 fractions (10–100 ng/ml), plumbagin (5 μM), apocynin (100 μM), or an appropriate vehicle of plumbagin. DPI could not be used in this method because of the interference of its vehicle. The reaction was started after the incubation period (10 min) by the addition of 100 μM NADPH and followed photometrically at 340 nm for 6 min at room temperature (20 ± 2°C). The NADPH oxidase activity was estimated from the area under the curve of absorbance values plotted versus time of the reaction period. The results were expressed as a percentage of the control reactions (where no procyanidin fractions or drugs were added).

GENERATION OF SUPEROXIDE ANIONS

In order to assess the possible direct superoxide scavenging properties of the procyanidin-rich fractions a superoxide generating system was needed. For this, superoxide anions were generated in the non-enzymatic PMS–NADH system and quantified by photometric measurements of the reduction product of NBT (Sigma–Aldrich), essentially following the procedure described by Alvarez et al. [2002] with some minor modifications. We used 600 μl test solutions made up in a phosphate buffer (50 mM KH_2PO_4-KOH, pH 7.4) and containing 166 μM β-nicotinamide adenine dinucleotide (NADH; Sigma–Aldrich), 43 μM NBT, and SOD (10 U/ml) in the

absence or in the presence of F4, F5, or F6 fractions at various concentrations (1–10 μg/ml).

The control experiments were carried out simultaneously without procyanidin fractions. In addition, the possible capacity of the fractions to directly reduce NBT was determined by adding them to solutions containing only NBT in phosphate buffer.

The reaction was started with test solutions already in the spectrophotometer (DU®730; Beckman Coulter) by adding PMS (final concentration of 3 μM, freshly diluted in the above phosphate buffer) and continued at room temperature for 5 min, a period in which absorbance increased linearly from the beginning. The rate of NBT reduction was calculated from the differential absorbance at 560 nm with respect to a blank solution in which PMS was replaced by a buffer solution, and was expressed as the increment of absorbance per min.

NADPH OXIDASE ACTIVITY IN INTACT HUVEC

The effects of procyanidin-rich fractions on NADPH oxidase activity were also tested in living cells. For these experiments, the HUVEC were placed at a concentration of 10,000 cells/well in 96-well microplates supplemented with endothelial cell growth medium. The cells were starved of serum and supplements for 12 h before the experiments and the resulting quiescent HUVEC were treated with different concentrations of F4, F5, or F6 fractions (50 ng/ml–1 μg/ml) or known NADPH oxidase inhibitors, while ROS production was measured at extracellular or intracellular levels as follows.

Extracellular superoxide formation was determined by the cytochrome c reduction method [Alvarez et al., 2010]. The serum and supplement-starved medium was removed from the HUVEC cultures and replaced by HBS supplemented with 1.5 mM $CaCl_2$ (calcium-rich HBS) and containing cytochrome c (200 μM) and NADH (100 μM), which were added to induce NADPH oxidase activation. The absorbance at 550 nm was monitored on a microplate reader (Multiscan Ex, Thermolabsystems) for 100 min at 37°C to obtain the $\Delta Abs_{550\,nm}$/min of each experiment. Superoxide production was calculated from the different cytochrome c reductions of the samples with or without SOD (100 U/ml), and the molar extinction coefficient for the change of ferricytochrome c to ferrocytochrome c (i.e., $\varepsilon 550\,nm = 21.1\,mM^{-1}/cm$). The flavoprotein inhibitor DPI (60 μM) was used in a series of experiments for assessing the NADPH oxidase dependency of the results. The F4, F5, or F6 fractions (50 ng/ml–1 μg/ml), SOD (100 U/ml), DPI (60 μM), or apocynin (100 μM) were added to the calcium-rich HBS at the same time as cytochrome c and NADH, so superoxide generation was measured in the presence of any of these compounds and in the appropriate control conditions.

Intracellular ROS production in the HUVEC was measured by c-DCFDA (Sigma–Aldrich) fluorescence using a fluorescence microplate reader (Fluostar Optima, BMG LABTECH, GmbH) [Alvarez et al., 2010]. The serum and supplement-starved medium from the HUVEC cultures was aspirated and the cells were washed with calcium-rich HBS and incubated in the dark for 20 min at 37°C in the presence of 5 μM of c-DCFDA for cell loading. The excess of c-DCFDA was washed away and the cells were placed into the chamber reader at 37°C. ROS generation was detected as a result of the oxidation of c-DCFDA (excitation 485 nm; emission 520 nm) preloaded in the HUVEC. Fluorescence was measured every 5 min for 2 h and the variation in relative fluorescence units (RFU) per min was calculated for each sample. The F4, F5, or F6 fractions (50 ng/ml–1 μg/ml), SOD (100 U/ml), DPI (60 μM), apocynin (100 μM), and plumbagin (5 μM) were added at the beginning of the fluorescence measurements without previous incubation. The known NADPH oxidase inhibitors were used in these experiments to compare their effects on NADPH oxidase inhibition with those from the procyanidin-rich fractions.

SUBCELLULAR PROTEIN FRACTIONATION

Expression distribution in the subcellular compartments of the main NADPH oxidase subunit in the endothelium (Nox4) was analyzed in the HUVEC model in culture. For this, HUVEC obtained as described above was cultured in 100 mm Petri dishes until confluence. Cells were detached with 0.25% (w/v) trypsin in 1 mM of EDTA (Gibco) and washed with phosphate buffer. After this, stepwise separation of subcellular protein fractions was obtained with a subcellular fractionation kit (Thermo Scientific, Rockford, USA) following the indications of the manufacturer. Briefly, specific reagents were sequentially added to the cell pellet and its derived fractions, which were stepwise settled by centrifugation to obtain cytoplasmatic, membrane, chromatin-bound, and cytoskeletal protein extracts of the cells. The first reagent selectively caused cell membrane permeabilization, releasing soluble cytoplasmatic contents. The second reagent solubilized plasma, mitochondria, and reticulum/golgi membranes, but not nuclear membranes. In the third step, we obtained the soluble nuclear extract and the chromatin-bound nuclear proteins. Finally, the recovered insoluble pellet was extracted to isolate the cytoskeletal proteins.

The protein extracts obtained were separated by sodium 12% SDS–PAGE and transferred to a polyvinylidene fluoride membrane for 45 min at 280 mA. The membranes were blocked for 2 h at room temperature with 5% skimmed milk in Tris-buffered saline–Tween-20 containing 20 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20. The membranes were then exposed with rabbit Nox4 antibody (1:650 dilution; Abcam) overnight, and then to peroxidase-conjugated goat anti-rabbit IgG (1:2,000). Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Ltd). In a series of experiments, the expression of glyceraldehyde-3-phosphate dehydrogenase (monoclonal mouse anti-GAPDH; Applied Biosystems; 1:1,000), a cytoplasmatic protein, VE-cadherin (monoclonal mouse anti-VE-cadherin, 1:1,000; Santa Cruz), a plasma membrane protein in the endothelium and vimentin (monoclonal mouse anti-vimentin; ready-to-use; Dako), a cytoskeleton protein, were also evaluated to assess the specificity of the subcellular extracts.

DATA ANALYSIS

The data are expressed as mean ± standard error of the mean (s.e.m.). Two-group comparisons were analyzed by Student's *t*-tests, as indicated in each case. Multiple comparisons were evaluated by ANOVA followed by Tukey tests. Statistical significance was considered for $P < 0.05$.

IC_{50} values of procyanidin fractions in each test were calculated by the least-squares linear regression, using a fitting analysis

TABLE I. Polyphenolic Profile of the Three Procyanidin Fractions (F4, F5, and F6) Obtained From Grape Pomace in Terms of the Main Constituent, the Percentage of Galloylation (%G), the Mean Degree of Polymerization (mDP), and the Oligomer Composition

Fraction	Main flavan-3-ol in procyanidin	%G	mDP	Composition
F4	Catechin	14	2.5	Dimers/trimers (3/2)
F5	Epicatechin	18	3.8	From dimers to heptamers. Mainly trimers (>50%)
F6	Epicatechin gallate	25	5.3	From dimers to octamers

mDP, total nmol/nmoles terminal units; %G, total galloylated nmols/total nmol.

program (Origin 6.0), of the linear part of the concentration–response curves obtained for the inhibitory effects of the fractions expressed as percentage of maximal response in each experimental protocol.

RESULTS

OBTAINING THE GRAPE PROCYANIDIN FRACTIONS

The aqueous fraction obtained as previously described was sub-fractionated in a Sephadex LH-20 column eluted in a gradient of methanol–water–acetone, rendering 6 fractions, 3 of which were selected for being practically devoid of monomers; these fractions, named F4, F5, and F6, were characterized in terms of their DP and %G as shown in Table I. The results showed that these fractions were mainly composed of oligomeric procyanidins with mDP from 2.5 to 5.3. Fraction F4 presented a 3/2 dimer/trimer proportion; fraction F5 was mainly composed of trimers (>50%), although some dimers to heptamers were detected, whereas dimers up to octamers were present in F6. The major oligomer constituents of the F4, F5, and F6 fractions were catechin, epicatechin, and epicatechin gallate, respectively, confirming the procyanidin nature of the fractions. The %G ranged from 14% to 25%. All of these results are summarized in Table I.

INHIBITION OF NADPH OXIDASE ACTIVITY IN HUVEC LYSATES

The activity of NADPH oxidase was measured in HUVEC lysates. In the lucigenin-dependent method, the chemiluminescence signal indicated the release of oxygen radicals, which increased linearly during the time of measurement after stimulation of NADPH oxidase with NADPH (100 μM; control response was 5163.3 ± 173.5 area under the curve from plotting relative luminescence units vs. time; n = 18). The mixture of NADPH (100 μM) and lucigenin (100 μM) in the test solution without cell lysates or the combination of HUVEC lysates and lucigenin in the absence of NADPH in the reaction did not induce an elevation in the chemiluminescence detected (data not shown), indicating that the reaction system did not auto-generate radicals.

Superoxide anion production was confirmed by the inhibition with SOD, an enzyme which specifically transforms superoxide to hydrogen peroxide. Similarly, DPI (100 μM), a well-known NADPH oxidase inhibitor, blocked all the NADPH-induced superoxide anion generation, confirming NADPH oxidase origin of the radicals measured.

In this lucigenin-enhanced chemiluminescence system, the three procyanidin fractions (100 ng/ml–5 μg/ml) inhibited NADPH oxidase activity in a concentration-dependent manner. The efficiency of the three fractions were similar (no statistical differences between them), as shown by the IC_{50} values of each fraction (Table II).

Similar results were obtained measuring NADPH oxidase activity by NADPH consumption in the presence of the procyanidin fractions. In this method, NADPH disappearance in the reaction mixture was photometrically monitored by 340 nm decay. The control reactions (without any inhibitory drug) displayed maximal NADPH consumption and they were considered as showing 100% NADPH oxidase activity (maximal control response was 1.029 ± 0.109 for the area under the curve from the plot of $Abs_{340\,nm}$ vs. time in minutes; n = 12). On the contrary, apocynin (100 μM) and plumbagin (5 μM) almost totally inhibited NADPH

TABLE II. Inhibition of NADPH Oxidase Activity in HUVEC Lysates and Scavenging of Superoxide Anions by F4, F5, and F6 Fractions and the Other Known Inhibitors

	NADPH oxidase activity		O_2^- Scavenging
	IC_{50} (μg/ml)[a] or % of activity[b]		IC_{50} (μg/ml)[a] or % of activity[b]
Fraction	Lucigenin-enhanced chemiluminescence	NADPH oxidation	PMS–NADH system
F4	3.4 ± 0.4	0.060 ± 0.006	>10.0*
F5	2.7 ± 0.3	0.062 ± 0.008	5.5 ± 0.4*
F6	2.5 ± 0.3	0.067 ± 0.008	8.0 ± 3.1*
DPI (100 μM)	0 (%)	–	–
PLUM (5 μM)	–	21.8 ± 12.1 (%)	–
APO (100 μM)	–	32.7 ± 15.7 (%)	–
SOD (10 U/ml)	–	–	29.1 ± 1.3 (%)

APO, apocynin; DPI, diphenyleneiodonium; PLUM, plumbagin; SOD, superoxide anion dismutase.
[a]Concentration required to achieve 50% inhibition under assay conditions; estimates ± s.e.m. were obtained by regression from dose–response curves.
[b]Percentage of control activity for the inhibitors at the concentration indicated.
*$P < 0.05$ with respect to value of the same fraction on NADPH oxidase activity.

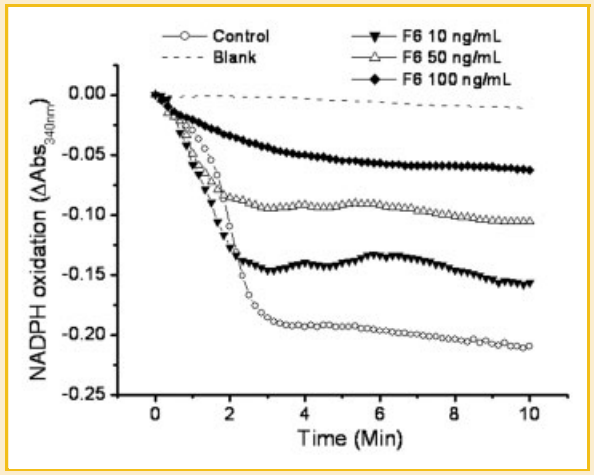

Fig. 1. NADPH oxidase activity and the effects of procyanidin fractions were measured in HUVEC lysates. NADPH oxidation was monitored as described in Materials and Methods Section. Representative time-course of NADPH consumption in the control and in the presence of different concentrations of F6 procyanidin fraction.

oxidase. The F4, F5, and F6 procyanidins fractions (10–100 ng/ml) inhibited NADPH oxidase activity in a concentration-dependent manner (Table II and Fig. 1).

DIRECT EFFECTS ON SUPEROXIDE ANION GENERATION

We used the non-enzymatic superoxide generation system PMS–NADH to assess the possibility of the antioxidant effects of the F4–F6 fractions being due to direct superoxide anion scavenging. In this system, superoxide anions were rapidly generated by the reaction between PMS and NADH and detected by NBT reduction (0.112 ± 0.004 $Abs_{560\,nm}$/min for control reaction; $n = 9$). The F4, F5, and F6 procyanidin fractions were incubated in this system in a wide range of concentrations: 100 ng/ml–10 μg/ml. However, only the highest concentrations of these fractions (2.5–10 μg/ml) were effective superoxide scavengers. Interestingly, 1 μg/ml of any of the procyanidin fractions had null effects on superoxide release, whereas at this concentration all of the fractions were proven to inhibit NADPH oxidase activity in the HUVEC lysates. This suggests that NADPH oxidase inhibition and superoxide scavenging of the procyanidin fractions are independent effects and that they can be distinguished by the concentration of the fractions. The IC_{50} values of each procyanidin fraction for the superoxide scavenging activity were significantly higher than those for NADPH oxidase inhibition (Table II).

NADPH OXIDASE ACTIVITY IN INTACT HUVEC

The NADPH oxidase activity was measured in living HUVEC cultures. Both extracellular and intracellular ROS production was measured in HUVEC cultures in the presence of the three procyanidin fractions. The effects of the F4, F5, and F6 fractions were compared with those from other known inhibitors of NADPH oxidase: apocynin, DPI, and plumbagin.

At the extracellular level, ROS production was measured by the cytochrome c reduction method (55.2 ± 6.8 O_2^- nM × min^{-1} × 10,000 $cells^{-1}$; $n = 6$, in control cultures). In this system, the three fractions, in a concentration-dependent manner and in a range of concentrations without superoxide scavenging properties (50 ng/ml–1 μg/ml), mostly inhibited ROS production (Fig. 2). Moreover, the effects of the F4–F6 fractions at the highest concentrations were similar to the effects of SOD (100 U/ml), the superoxide anion metabolizing enzyme, and apocynin (100 μM) and DPI (60 μM), two well-known inhibitors of the NADPH oxidase complex (Fig. 2). No significant differences were observed between the efficiency of the three fractions, although F6 was the most effective.

Intracellular ROS production was measured by the fluorescence variation of c-DCFDA pre-loaded in HUVEC cultures (165.2 ± 3.2 RFU/min for control; $n = 5$). The F4, F5, and F6 fractions were efficient inhibitors of ROS production, in a concentration-dependent manner (50 ng/ml–1 μg/ml). Again, this effect appeared at a concentration range without superoxide scavenging properties, suggesting a NADPH oxidase inhibitory action for all of the fractions also at intracellular levels (Fig. 3, Panels a–c). There were no differences between the efficiency of the three fractions.

The effects of the F4, F5, and F6 fractions (500 ng/ml) were compared with the effects of well-known NADPH oxidase inhibitors, such as apocynin, DPI, and plumbagin at their maximal concentrations, resulting in a similar effectiveness for apocynin 100 μM and the three fractions and a slightly pronounced inhibition for DPI 60 μM and plumbagin 5 μM, which were the NADPH oxidase inhibitors who achieved the highest inhibition in the conditions of the experiments ($59.47 \pm 1.09\%$ vs. $19.90 \pm 2.42\%$ of the control for DPI and plumbagin, respectively; $P < 0.05$ between them; $n = 5$; Fig. 3, Panel d). On the contrary, SOD (100 U/ml) was not an effective inhibitor in these experiments compared with the control ($98.01 \pm 3.31\%$ of the control; $P > 0.05$ with respect to the control; $n = 3$), probably because it is not able to penetrate the cell membrane and reach the intracellular space due to its large size (Fig. 3, Panel d).

NOX4 EXPRESSION LEVELS IN ENDOTHELIAL SUBCELLULAR FRACTIONS

The main NADPH oxidase subunit expressed in the endothelium, Nox4, was analyzed in terms of its distribution in the subcellular fractions. Four different protein extracts were obtained from HUVEC: cytoplasmatic, membrane, chromatin-bound, and cytoskeletal extracts. Nox4 expression in each protein fraction was studied by SDS–PAGE, and subsequent Western blot. Results showed Nox4 localization mainly in cytoplasmatic and membrane extracts, with sporadic expression in chromatin-bound extracts, and no detection in cytoskeletal extracts. It means that in all the experiments, Nox4 expression was detected in the cytoplasmatic and membrane extracts, but not always Nox4 was present in the chromatin-bound extracts (Fig. 4). No apparent reason was found to this intermittent result in the nuclear extract, but it seems that Nox4 location at nucleus is governed by a dynamic equilibrium.

HUVEC VIABILITY

The MTT system is a simple, accurate, and reproducible means of measuring the activity of living cells. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of MTT solubilized

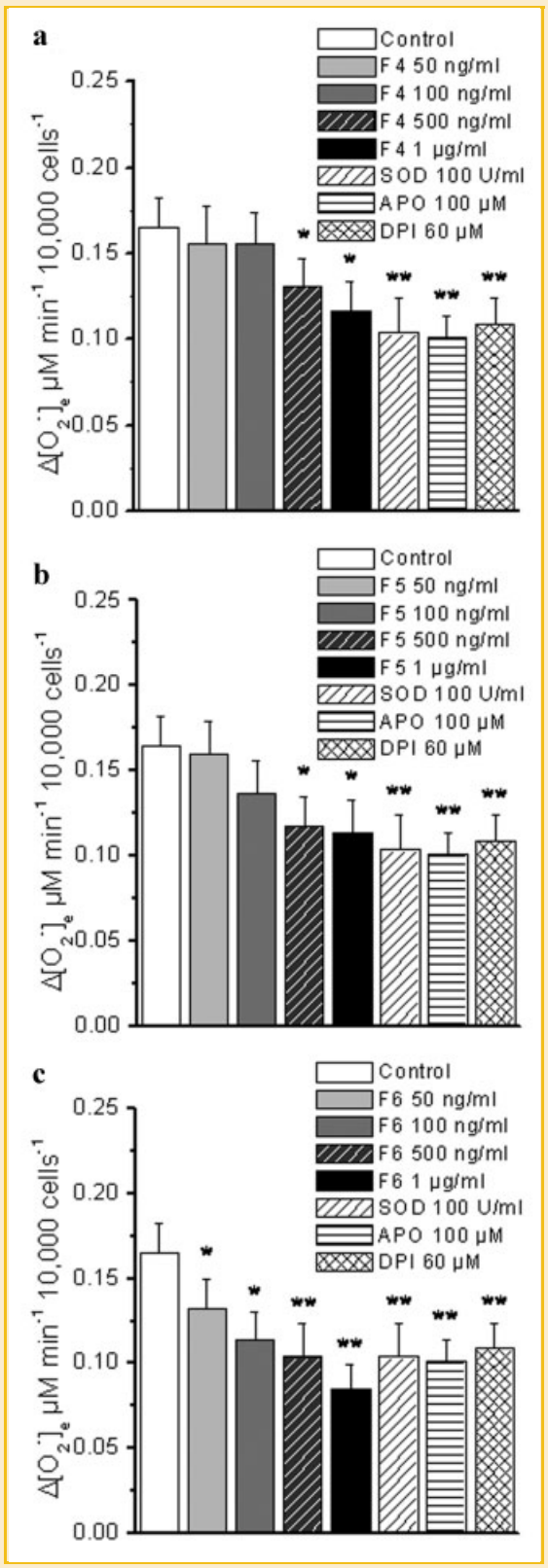

Fig. 2. Effects of different concentrations (50 ng/ml–1 µg/ml) of F4 (a), F5 (b), and F6 (c) procyanidin fractions on NADH 100 µM-induced extracellular superoxide production in confluent HUVEC cultures. Comparison with the effects of SOD, apocynin (APO), and DPI. Columns represent mean values of at least three experiments ± s.e.m. shown as error bars. $^{*}P < 0.05$; $^{**}P < 0.01$ with respect to the control as evaluated with ANOVA followed by Tukey test.

in tissue culture media, yielding purple MTT formazan crystals, which, once dissolved, can be measured spectrophotometrically. An increase in viable cell number results in an increase in absorbance (maximal absorbance for control HUVEC cultures was 0.457 ± 0.004 $Abs_{560\,nm}$; n = 6).

The results indicate that after 6 h of treatment, cell viability was enhanced in the presence of complete medium compared to the control (HUVEC in deprived medium; Fig. 3). The three procyanidin fractions at the concentrations tested (100 ng/ml–5 µg/ml) did not modify the integrity and viability of the HUVEC compared to the control. Also, apocynin (100 µM) and plumbagin (5 µM) did not show any effect compared to the control cultures in deprived medium. On the contrary, DPI (60 µM) showed a statistically significant reduction of cell viability, indicating a toxic effect on the HUVEC (Fig. 5).

DISCUSSION

We showed for the first time that procyanidin fractions from grape pomace inhibited NADPH oxidase activity on HUVEC lysates in a concentration-dependent manner up to 1 µg/ml. This effect was independent of the superoxide anion scavenging activity of the fractions, evidenced only at concentrations of 2.5 µg/ml and higher. Moreover, the three fractions were effective inhibitors of NADPH oxidase in living HUVEC cultures, being active at both the extra- and the intracellular level. Therefore, we obtained hydrophilic procyanidin fractions from grape pomace with NADPH oxidase inhibitory activity at the human endothelial level.

The NADPH oxidase activity was analyzed in cell lysates from HUVEC cultures by two different methods. In the lucigenin-enhanced chemiluminescence method, superoxide production was demonstrated by inhibition with SOD, the specific superoxide anion metabolizer enzyme, whereas the NADPH oxidase origin of these anions was confirmed by DPI inhibition, a known selective NADPH oxidase inhibitor. All of the three grape procyanidin fractions were effective inhibitors of NADPH oxidase under these conditions. Their activities were in a concentration-dependent manner in the range of 100 ng/ml to 5 µg/ml. In the NADPH oxidation method, the procyanidin fractions (F4–F6) also inhibited NADPH oxidase in a concentration-dependent manner, but at lower concentrations than in the lucigenin-enhanced chemiluminescence method. This was possible because the former method is a more direct one: NADPH oxidation is directly denoted by its consumption by the oxidase, whereas the lucigenin-based method requires the reaction between O_2^- and lucigenin prior to detection. In any case, these results agreed that procyanidin fractions contain active molecules with NADPH oxidase inhibition activities. However, this activity could not be related to the DP, the %G, or even the oligomer composition of each fraction because no differences were observed between the inhibitory activities of the fractions.

Considering the superoxide anion scavenging properties observed previously for other products derived from grapes [Garcia-Alonso et al., 2005], we tested this hypothesis using the PMS–NADH system in which the chemical reaction between PMS and NADH spontaneously produces superoxide anions. Under these conditions,

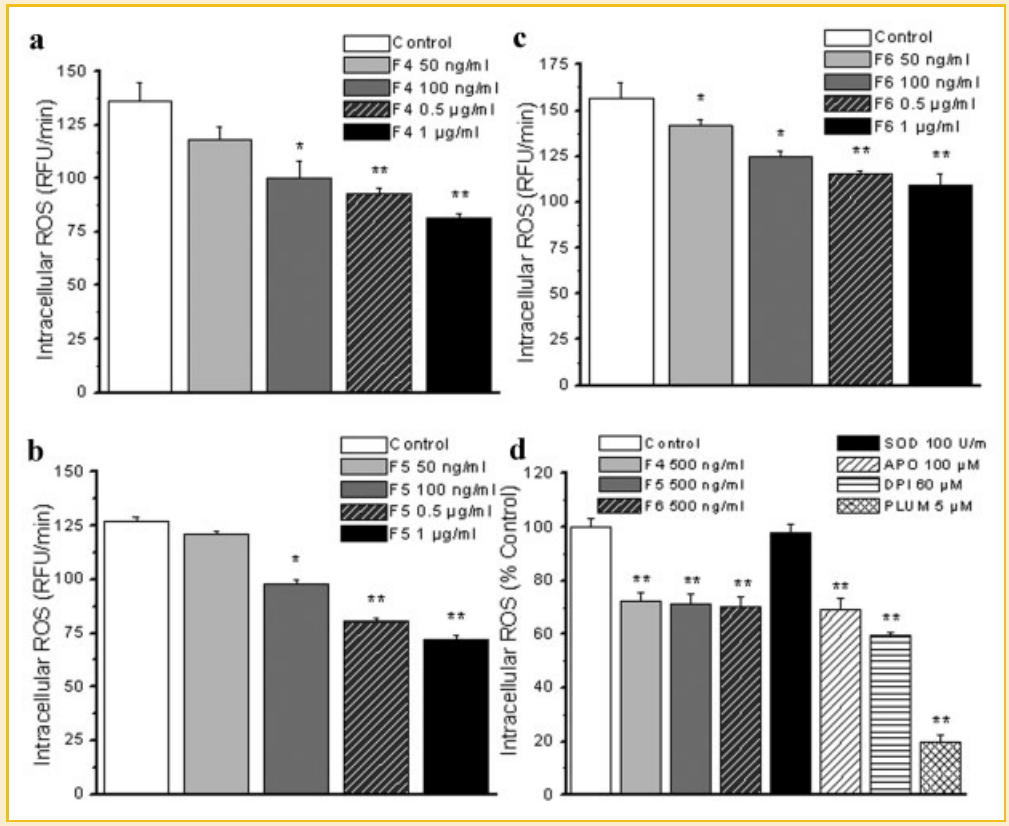

Fig. 3. Intracellular ROS production in c-DCFDA pre-loaded HUVEC cultures in the presence of different concentrations of F4 (a), F5 (b), and F6 (c) procyanidin fractions represented as relative fluorescence unit (RFU) increments per min. d: Comparison of the NADPH oxidase inhibition effects on intracellular ROS production by the F4, F5, and F6 fractions and SOD, apocynin (APO), DPI, and plumbagin (PLUM) in the concentrations indicated and represented as percentage of the control. Columns represent mean values of at least three experiments ± s.e.m. shown as error bars. $^{*}P < 0.05$; $^{**}P < 0.01$ with respect to the control as evaluated with ANOVA followed by Tukey test.

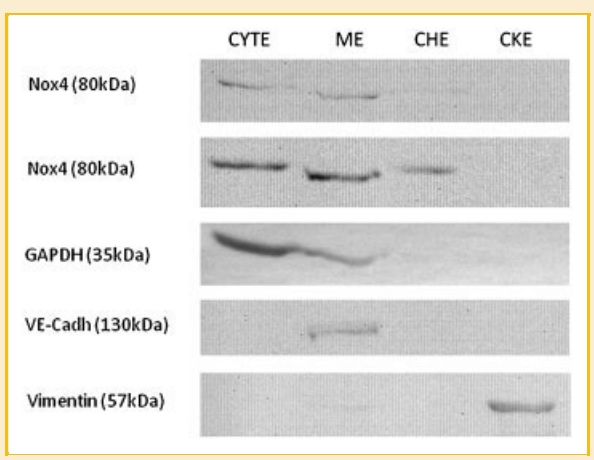

Fig. 4. Representative Western blots showing subcellular distribution of Nox4 in HUVEC. Columns refer to the cytoplasmatic (CYTE), membrane (ME), chromatin-bound (CHE), or cytoskeletal (CKE) extracts from HUVEC. Nox4 was found always in the cytoplasmatic and the membrane extracts, but in some experiments it also appeared in the chromatin-bound extract, as shown in the second plot. GAPDH, a cytoplasmatic protein, VE-cadherin (VE-Cadh), a typical membrane protein in endothelial cells, and vimentin, a component of cytoskeleton, were used to test the specificity of the subcellular extracts.

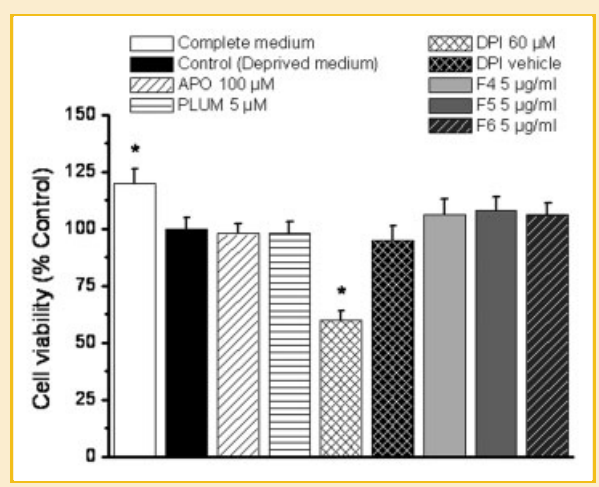

Fig. 5. HUVEC viability measured via mitochondrial dehydrogenase activity (MTT method). Comparison of the results after 6 h of incubation with complete medium, F4, F5, and F6 fractions, apocynin (APO), DPI, vehicle of DPI, and plumbagin (PLUM) in the concentrations indicated and represented as percentage of the control (HUVEC in medium deprived of serum and supplements). Columns represent mean values of at least three experiments ± s.e.m. shown as error bars. $^{*}P < 0.05$ with respect to the control as analyzed by Student's *t*-test.

all of the three fractions showed superoxide anion scavenging activities, but at concentrations higher than 2.5 μg/ml, while the lower concentrations were mostly ineffective. Furthermore, the IC_{50} values of each fraction for NADPH oxidase inhibition were always significantly lower than the corresponding values for superoxide scavenging. These data indicated that the NADPH oxidase inhibitor activity observed with the fractions at concentrations up to 1 μg/ml was independent of the scavenging effect, which was only evidenced at concentrations higher than 2.5 μg/ml. These results definitively confirmed the NADPH oxidase inhibitory ability of our procyanidin fractions in the in vitro experiments.

Not all of the NADPH oxidase isoforms are expressed equally in human endothelial cells. In fact, the expression of the Nox4 homolog markedly exceeds that of other Nox proteins, including Nox2 [Ago et al., 2004; Van Buul et al., 2005; Alvarez et al., 2010]. Nox4 expression in our HUVEC model has been also detected by immunofluorescence [Alvarez et al., 2010]. This suggests that our assay for NADPH oxidase activity in HUVEC lysates would be focused on the Nox4 isoform of NADPH oxidase, although the presence of other isoforms cannot be discarded. Therefore, we cannot conclude that the inhibitory activity observed for the F4–F6 fractions was specific for the Nox4 isoform, but these fractions inhibited at least this NADPH oxidase isoform. The expression analysis of Nox4 in the subcellular fractions from HUVEC revealed that this enzyme was present mainly in the cytoplasmatic and in the membrane protein fractions, and, circumstantially, in the chromatin-bound fraction, but never in the cytoskeletal fraction. These data partially agree with previously reported by Kuroda et al. [2005], who found Nox4 in the nucleus of HUVEC. However, in our cell model, this is not the preferential location for Nox4, since the presence of Nox4 at nucleus was sporadic. The mechanisms regulating this translocation and the function of Nox4 at this location need further investigations, but they were not the aims of our study. Our data better agree with the recent observations of Block et al. [2009], who situated Nox4 in the membranes and in the mitochondria of glomerular mesangial cells. In fact, this distribution of Nox4 correlates better with the situation of resting and/or quiescent cells, as in our experiments. Cells under stimulation could express Nox4 in the nucleus, probably modulating the expression of some genes.

The NADPH oxidase inhibitory activity for procyanidins has not been described previously. However, it has been recently reported for some flavanol metabolites [Steffen et al., 2008]. The catechin monomers [(−)-epicatechin and (+)-catechin], despite their superoxide scavenging properties, did not inhibit NADPH oxidase activity in endothelial cells [Steffen et al., 2008]. Interestingly, the metabolites 3′- and 4′-*O*-methyl epicatechin or COMT-methylated epicatechin presented the reverse pattern. Other epicatechin-related products such as epicatechin glucuronide and procyanidin B2 were able to scavenge superoxide anions and to inhibit NADPH oxidase. An apocynin-like mode of NADPH oxidase inhibition is suggested by the authors for these compounds [Steffen et al., 2008]. However, these results were mainly from catechin monomers, whereas the major composition of our fractions was procyanidin oligomers, and the antioxidant activity of flavanols differs from that of their procyanidins [Yilmaz and Toledo, 2004].

Once the inhibitory action of the procyanidin fractions on NADPH oxidase activity was demonstrated in vitro, our next objective was to study the effects of the fractions on the NADPH oxidase activity in living HUVEC cultures. Extracellular superoxide production generated by HUVEC cultures and detected by cytochrome c reduction was equally inhibited by all of the procyanidin fractions (F4, F5, and F6) in a concentration range up to 1 μg/ml. These effective concentrations were in the non-scavenging range defined above. In the same way, the intracellular ROS production detected with c-DCFDA was equally inhibited in a concentration-dependent manner by the three procyanidin fractions, also at non-scavenging concentrations (up to 1 μg/ml). These results also indicated that the NADPH oxidase inhibitors present in the F4–F6 fractions can act at the intracellular level, probably stimulating some signaling pathway leading to NADPH oxidase inhibition. Moreover, the effectiveness of the three fractions was similar between them and comparable to the inhibitions obtained with the maximal concentrations of the other known NADPH oxidase inhibitors, such as apocynin and DPI.

Taken altogether, these results revealed that F4–F6 procyanidin fractions of grape pomace were effective NADPH oxidase inhibitors in a living cell system. Importantly, the fractions achieved the same level of activity that the other known NADPH oxidase inhibitors mentioned. However, an important difference between the F4–F6 fractions and the other inhibitors exists concerning their hydrophilic properties. All of the three fractions were perfectly soluble in water at the concentrations tested, whereas all of the other drugs (apocynin, DPI, and plumbagin) were less hydrophilic. This could be an important issue for drug development from grape procyanidins. Furthermore, the cell viability experiments revealed that the three procyanidin fractions respected cell integrity and viability up to 5 μg/ml. By contrast, the main NADPH oxidase inhibitor, DPI, showed a marked toxic effect on the HUVEC cultures. Therefore, procyanidin fractions could serve as an alternative source of new NADPH oxidase inhibitors with better responses in vascular cells than previously studied inhibitors such as apocynin [Heumuller et al., 2008].

Despite the different polyphenol profiles of the three procyanidin fractions, the effectiveness of NADPH oxidase inhibition was not statistically different between them. Therefore, we can conclude that grape pomace procyanidins made up of catechin, epicatechin, or epicatechin gallate, with 14–25% galloylation and 2.5–5.3 mDP, were equally effective at endothelial NADPH oxidase inhibition. This also means that the dimers and trimers of procyanidins were probably the main molecules responsible for this activity since they are the unique components common to all three fractions. In fact, it has been suggested that antiradical activity increases with mDP from 2 to 5, and that it decreases at higher levels of polymerization [de Gaulejac et al., 1999]. On the other hand, it was previously proven that galloylation can improve the antioxidant activity of procyanidins [Tourino et al., 2005], so %G could also explain the antioxidant potency of the fractions. The other parameter, the major flavanol constituents, did not seem to be very important in the effectiveness of NADPH oxidase inhibition.

The NADPH oxidase inhibition activity described in our work for procyanidins from grape pomace has not been reported before,

despite the inhibitor activity on this oxidase having been described for other components of grapes [Orallo et al., 2002; Chow et al., 2007; Castilla et al., 2008]. Furthermore, polyphenols from grape extracts, sometimes with a high content of procyanidins, have been demonstrated to reduce NADPH oxidase subunit expression in human neutrophil mononuclear cells and in an endothelial cell line [Davalos et al., 2009]; and at the left ventricle in a model of high-fructose-fed rats [Al-Awwadi et al., 2005] and in an obese hamster model [Decorde et al., 2009]. These effects on NADPH oxidase expression could have contributed to the direct NADPH oxidase inhibitory activity observed in our in vivo treatments of grape procyanidins. Various data indicate that activation of NADPH oxidase and the production of ROS induce an autoamplification loop, leading to an increased expression of the enzyme [Cai et al., 2003], whereas inhibitors of the activity of this enzyme reduce its expression. Consequently, it is possible that procyanidins modulate the expression of NADPH oxidase by inhibiting its activity and scavenging ROS.

CONCLUSIONS

In conclusion, we used a readily available by-product of the wine industry, distilled grape pomace, to obtain three differently polymerized and galloylated procyanidin fractions. We reported for the first time that these procyanidins are inhibitors of NADPH oxidase activity in human endothelial cells of in vitro and ex vivo models. This effect was independent of any superoxide scavenging property and the major flavanol monomer constituent, the mDP and the %G of the procyanidins fractions studied. Therefore, our results will aid in the search for a new source of active NADPH oxidase inhibitors at the human endothelial level which achieved the same effects that known NADPH oxidase inhibitors, such as DPI or apocynin, but with a better hydrophilicity and safety than DPI. Therefore, grape procyanidins could be a new source for therapeutic alternatives for achieving a reduction of the oxidative stress associated with cardiovascular and/or inflammatory diseases related to NADPH oxidase hyperactivity.

ACKNOWLEDGMENTS

The authors are grateful to anonymous donor mothers and the staff of the Births Unit of *Hospital Clínico Universitario de Santiago de Compostela* for their collaboration in the collection of umbilical cords for HUVEC cultures. We thank Noelia Crespo for excellent technical assistance. Thanks to *Aguardientes de Galicia, S.A.* (Vedra, A Coruña, Spain) for providing grape pomace. The *Isidro Parga Pondal* programme from *Xunta de Galicia* (Spain) supported the work of E. Alvarez. The Ministry of Science and Technology and *Xunta de Galicia* supported part of the research with Projects AGL2006-12210-C03-03 and 2007/PX046, respectively.

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