DOI 10.1002/mnfr.200800134 Mol. Nutr. Food Res. 2009, *53*, 266 – 276

Research Article

Oligomeric procyanidins inhibit cell migration and modulate the expression of migration and proliferation associated genes in human umbilical vascular endothelial cells

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The consumption of flavan-3-ols has been associated with reduced risk of cardiovascular diseases and improvements in vascular function. However, the nature of the flavan-3-ols responsible and the mechanisms underlying the vascular responses are not fully understood. We used microarrays to search for molecular changes in response to the exposure to (−)-epicatechin (EC), procyanidin dimer B2, and a mixture of oligomeric procyanidins in human umbilical vein endothelial cells (HUVECs). No gene expression changes were detected in HUVECs exposed to EC or dimer B2, however, the oligomeric procyanidins induced significant gene expression changes in both resting and TNF-α-stimulated cells. In particular, the expression of genes such as *ADAMTS1*, *THBS1*, *ANGPT2*, *CYR61*, *ET-1*, *EDG3*, and *PDE4B* involved in endothelial cell migration and proliferation, were substantially over-represented. Also, exposure to the oligomers arrested the cells at the G_0/G_1 phase and inhibited cell migration. These data show that human endothelial cells respond to oligomeric procyanidins by exhibiting a less migratory phenotype and by a general modulation of the expression of genes that are associated with key events in the angiogenic process. The molecular changes associated with procyanidin treatment identified in this study are consistent with the beneficial effects of flavan-3-ols on vascular function.

Keywords: Cell migration / Endothelium / HUVECs / Microarray / Procyanidins

Received: March 31, 2008; revised: May 19, 2008; accepted: May 28, 2008

1 Introduction

The endothelium plays a pivotal role in the regulation of vascular functions such as modulation of vascular tone, adhesion properties, inflammation, thrombotic balance, and cell proliferation. Alterations in these functions are associated with the development of cardiovascular diseases (CVDs) [1]. Endothelial function is regulated by endogenous factors such as oxidized low density lipoprotein (LDL) and cytokines and by exogenous factors such as diet-derived components that can have important modulatory effects [2]. Epidemiological, *in vitro*, animal and dietary

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Abbreviations: ACE, angiotensin I converting enzyme (peptidyl-dipeptidase A) 1; **ADAMTS1,** ADAM metallopeptidase with thrombospondin type 1 motif, 1; **ANGPT2,** angiopoietin 2; **BMP4,** bone morphogenetic protein 4; **CTGF,** connective tissue growth factor; **CVDs,** cardiovascular diseases; **CXCR4,** chemokine (C-X-C motif) receptor 4; **CYR61,** cysteine-rich, angiogenic inducer, 61; **dp,** degree of polymerization; **EC,** (−)-epicatechin; **EDG3,** endothelial differentiation, sphingolipid G-protein-coupled receptor, 3; **eNOS,** endothelial nitric oxide synthase; **ET-1,** endothelin-1; **FDR,** false discovery rate; **FLT1,** fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor); **HUVECs,** human umbilical vein endothelial cells; **IPA,** Ingenuity Pathways Analysis; **KITLG,** KIT ligand; **KLF4,** Kruppel-like factor 4 (gut); **MMP,** matrix metalloproteinases; **PDE4B,** phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila); **THBS1,** thrombospondin 1; **VEGF,** vascular endothelial growth factor

intervention studies indicate that the consumption of flavan-3-ol-rich foods and beverages is associated with protective effects against vascular dysfunction and prevention of CVDs [3]. Several human studies have shown improvements in biomarkers of endothelial function after consumption of flavan-3-ol-rich beverages such as tea, cocoa or red wine [4]. For example, it has been reported that in subjects with smoking-related endothelial dysfunction, the consumption of a flavan-3-ol-rich cocoa drink increased flow-mediated dilation (FMD) in a dose-dependent manner [5]. Also, in hypercholesterolemic postmenopausal women, chronic consumption of flavan-3-ol-rich cocoa improved endothelial function, and decreased plasma soluble vascular cell adhesion molecule-1 [6]. In addition to tea, cocoa, and red wine, flavan-3-ols are also abundant in apples, berries, grapes, and some herbal remedies. The flavan-3-ols include the monomers (+)-catechin and (−)-epicatechin (EC), galloylated/gallated derivatives (*e.g.*, epigallocatechin gallate, (EGCG), the major flavonoid in green tea), and procyanidins which are oligomers/polymers of EC and/or (+)-catechin units. Flavan-3-ols and (or) procyanidins may modulate endothelial function *via* several mechanisms: *e.g.*, improving lipid balance and lipoprotein metabolism, increasing the production and release of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) causing vasodilation and preventing platelet adhesion, inhibiting angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 (ACE), inhibiting endothelin-1 (ET-1) synthesis, inhibiting 2,4-dienoyl CoA reductase 1, mitochondrial (NAD(P)H) oxidase and, increasing the release of prostacyclins [3, 7].

The development of atherosclerotic lesions is associated with an accelerated formation of new blood vessels (angiogenesis) [8]. Angiogenesis is a complex process characterized by the early degradation of the extracellular matrix (predominantly by matrix metalloproteinases (MMPs)), followed by migration and proliferation of endothelial cells and maturation of new blood vessels, and occurs in response to many different factors [9]. Several *in vitro* and *in vivo* studies show that polyphenols present in red wine or green tea can inhibit key events in the angiogenic process such as the proliferation and migration of endothelial cells, and modulate the expression and activity of pro- and (or) anti-angiogenic factors [10]. The flavan-3-ol-rich extracts used in many reported studies are usually a mixture of monomers and oligomers (and also of some other polyphenols) and the observations do not provide information as to the nature of the bioactive components [11, 12]. Other reports allude to the cardiovascular protective effects of purified fractions enriched in a particular flavan-3-ol. In general, these reports show that oligomeric procyanidins of higher degree of polymerization (dp) are usually more active than their mono- and dimeric counterparts [13, 14].

Many of the reported activities of flavan-3-ol-rich foods and extracts would impact favorably to reduce cardiovascular risk factors and support the notion that flavan-3-ols (including procyanidins) are a promising class of compounds for cardiovascular protection. However, the nature of the flavan-3-ols responsible and the molecular mechanisms underlying the vascular responses remain unclear. In the present study, we investigated the changes in patterns of gene expression induced by monomeric flavan-3-ols and oligomeric procyanidins in both unstimulated and TNF-α-stimulated human umbilical vein endothelial cells (HUVECs). The data obtained indicated that exposure of cells to a mixture of oligomeric procyanidins with a mean dp of 3.9 (dp 3.9) altered significantly the expression of many transcripts, whereas treatment with monomeric EC or procyanidin B2 had little effect on gene expression. Further analysis of the transcriptomic data to identify the key biological functions affected by the procyanidin fraction indicated an over-representation of genes associated with cell growth and proliferation, cell movement, cell death, and cardiovascular system development. Direct assessment of whole cell functional changes in response to treatments confirmed that the transcriptional changes corresponded with changes at the cellular level. The data presented here show that oligomeric procyanidins inhibit migration of HUVECs and modulate the expression of genes that are involved in the angiogenesis process. These effects on vascular endothelial cells are in keeping with the cardioprotective effects of flavan-3-ols and may represent an important mechanism of action for these dietary compounds.

2 Materials and methods

2.1 Materials

The procyanidin-enriched fraction was purified from a cider apple (*Malus domestica*, variety Antoinette). Flavanol oligomers/polymers were the only procyanidins present in the fraction with EC the main flavanol unit (>95%) [15]. The mean dp was 3.9 ± 0.1 ($n = 3$) as determined by a quantitative thiolysis-HPLC method [16]. The dp 3.9 fraction was further characterized using a semi-quantitative normal phase HPLC with fluorescence detection method which indicated that the oligomer size distribution of the extract was approximately: 15% flavanol dimers, 55% oligomers of dp 3 – 7, 24% of polymers of dp 8 – 10, and 5% polymers with dp > 10 with monomers present only in trace amounts [17]. A stock solution of the extract was prepared by dissolving the test sample in DMSO (15 mg/mL). Purified EC and procyanidin dimer B2 were purchased from Extrasynthese (Genay, France) and were also dissolved in DMSO. Recombinant human TNF-α from R&D Systems (Abingdon, UK) was dissolved in PBS containing 1% BSA.

2.2 Cell culture and cell treatments

HUVECs (Cambrex Bio Science, Wokingham, UK) were grown in endothelial growth media 2 (EGM2) Bullet Kit

(Lonza-Cambrex) and maintained at 37°C under a 5% CO_2/95% air atmosphere at constant humidity. Cells (passage number 2–4, doubling population ≤10) were seeded onto six-well plates at a density of 3500 cells/cm^2 and treated on day 5 after seeding (confluent monolayers) as follows: (i) EC (5 and 25 μM), 4 h; (ii) procyanidin dimer B2 (0.5 and 2.5 μM), 4 h; (iii) dp 3.9 fraction (10 μg/mL, estimated to be ~8.9 μM based on an average Mw for the dp 3.9 fraction = 1125.2 g/mol; Mw = 290 × *n* – ((*n* – 1) × 2) (*n* = chain length)), 4 h; (iv) dp 3.9 fraction (10 μg/mL) for 45 min followed by TNF-α (10 ng/mL) for 6 h (pre-cotreatment). Control cells were treated accordingly with DMSO (<0.1% in the culture medium). For this study, we selected one concentration (5 μM) of EC that is within the reported range in human plasma [18] and a second five-fold higher concentration that may be achieved after consumption of a flavanol enriched product [19]. For the procyanidin dimers we used two concentrations that were between those reported for total procyanidin dimers from a high dose in rat plasma (~2.5 μg/mL equivalent to 4.3 μM [20]) and for dimer B2 from a regular dietary dose in human plasma (0.04 μM [19]). For the dp 3.9 procyanidins we selected a total concentration of 10 μg/mL similar to that recently reported in the plasma of rats fed apple procyanidins [20].

2.3 RNA extraction

Total RNA was isolated from control and treated cells using an RNeasy® Mini Kit (Qiagen). RNA concentration and purity were checked using the Nanodrop spectrophotometer ND-1000 (LabTech International, UK) measuring the optical densities at 260 and 280 nm. Only samples with a 260:280 ratio between 1.8 and 2.1 were used for microarray experiments. In a preliminary study and for treatments with EC (5 and 25 μM) or dimer B2 (0.5 and 2.5 μM), we created control and treated "pooled" groups by mixing the RNA from three separate RNA preparations, each from an independent dish of cells. Each pooled sample was hybridized on a single chip. In a second set of experiments, treatments with EC (25 μM) or dp 3.9 oligomers fraction (8.9 μM), RNA extractions and hybridizations were all performed separately in triplicate.

2.4 Microarrays analysis

Total RNA (5 μg) was used for double-stranded cDNA synthesis and generation of biotin-labeled cRNA, following the manufacturer's one-cycle protocol (Affymetrix). The quality and size of fragmented biotin-labeled cRNAs was assessed with the Agilent Bioanalyzer 2100 prior to hybridization onto Human Genome U133 Plus 2.0 chips (http://www.affymetrix.com/products/arrays/specific/hgu133plus.affx). Fluorescence intensity was captured with a laser GeneChip® Scanner 3000. Affymetrix Gene Chip Operative software with the Expression Default Settings was used for data acquisition and quantification. Microarrays data (.CEL files) were normalized by the Robust Multi-array Average (RMA) method. Normalized data were imported into the GEPAS software (Gene Expression Pattern Analysis Suite; http://gepas.bioinfo.cipf.es/, Bioinformatics Department of the Research Centre Principe Felipe, Valencia, Spain) for statistical differential gene expression analysis between treated and control cells. In those experiments performed with pooled RNA samples, genes were determined to have altered expression between treated and control cells when they met the two following criteria: (i) probes exhibited a change ≥1.5 or ≤0.67 (ratio treated/control) at the two concentrations tested; (ii) in cases where a gene is represented by several probes, two probes or more exhibited a change in the same direction. Statistical differential gene expression analysis between control and treated cells (EC, 25 μM or dp 3.9, 8.9 μM) was performed by unpaired *t*-test and the false discovery rate (FDR: expected proportion of false positives among the declared significant results) was controlled by Benjamini–Hochberg adjustment and implemented with the T-Rex tool of the GEPAS package. Minimum information about a microarray experiment (MIAME) compliant data from control and treated HUVECs after exposure to the oligomeric fraction in both resting and cytokine-stimulated cells have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE9647.

Datasets containing the significantly regulated genes by dp 3.9 in resting and stimulated cells were uploaded into the Ingenuity Pathways Analysis (IPA) software (http://www.ingenuity.com) in order to identify significantly altered biological networks and genes potentially involved in specific functions and pathways. This web-delivered application uses a knowledge base derived from the literature to relate gene products with each other, based on their interaction and function. To further confirm and identify biological and functional meaning of the microarrays results, we used Babelomics (http://babelomics.bioinfo.cipf.es/cgi-bin/tools.cgi) which is part of the GEPAS suite and includes the FatiScan tool that finds blocks of functionally related genes, showing a significant asymmetric distribution toward the extremes of the complete list of probes displayed in the microchip, and ranked by the *t* statistics for differential expression between treated and control cells. It uses a segment test (two tailed Fisher's exact test; 30 partitions; threshold *p*-value, 0.05; human repository Gene Ontology (GO) biological process; only deepest significant terms) to select significantly over-represented functional terms associated with genes most induced in treated cells *versus* control cells. All the steps for gene expression and functional analysis are summarized in a flow diagram (Fig. 1).

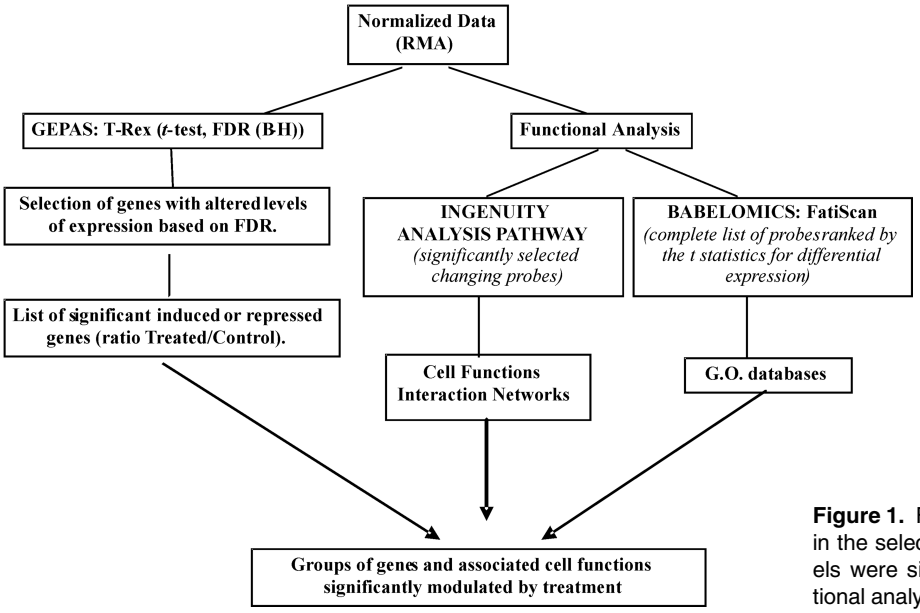

Figure 1. Flow diagram indicating major steps in the selection of genes whose expression levels were significantly modulated, and the functional analysis strategy.

2.5 RT-PCR

Changes in the expression of eight selected genes were assessed by one-step quantitative RT-PCR (Taqman system, Applied Biosystems, ABI). Amplification was performed using TaqMan Universal Master Mix and primers and probes selected from Assays-on-demand (ABI): ADAM metallopeptidase with thrombospondin type 1 motif, 1 (*ADAMTS1*), Hs00199608_m1; angiopoietin 2 (*ANGPT2*), Hs00169867_m1; cysteine-rich, angiogenic inducer, 61 (*CYR61*), Hs00155479_m1; endothelial differentiation, sphingolipid G-protein-coupled receptor, 3 (*EDG3*), Hs01019574_m1; *ET-1*, Hs00174961_m1; fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) (*FLT1*), Hs01052936_m1; phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila) (*PDE4B*), Hs00387320_m1; and thrombospondin 1 (*THBS1*), Hs00170236_m1 (_m indicates an assay whose probe spans an exon junction and will not detect genomic DNA). The one-step RT-PCR reactions were performed on an ABI prism 7700 system following the manufacturer's thermal conditions. All assays for a particular gene were undertaken at the same time under identical conditions and in triplicate. The expression levels of target genes were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs99999905_m1) utilizing a standard curve method for quantification.

2.6 Measurement of human ET-1 release

Cell culture supernatant from control and treated cells were collected and the production of ET-1 was determined with the TiterZyme Enzyme Immunometric Assay (Assay Designs, Cambridge BioSciences, Cambridge, UK), that uses a rabbit polyclonal antibody against human ET-1, following the manufacturer's instructions. After the incubation, washing and labeling steps, absorption was measured at 450 nm in a microtiter plate reader (ELx800, Bio-Tek-Instruments, UK). Measurements were done in triplicate and results represent mean value ± SD.

2.7 Cell proliferation and viability

At the end of each treatment, cells were harvested by Clonetics® Trypsin (0.025%)/EDTA (0.01%) (Cambrex) and resuspended in culture medium containing 10% FCS (fetal calf serum). The cell suspension was incubated with an equal volume of Trypan blue and cells were counted using a dual-chamber haemocytometer. Results of proliferation and viability in treated cells are presented in comparison to control cells. Data are presented as mean values ± SD from triplicate experiments.

2.8 Cell cycle analysis

To analyze cell cycle distribution, control and treated HUVECs (2×10^5) were trypsinized and collected after exposure to the dp 3.9 extract, fixed in ice-cold ethanol/PBS (70:30) for 2 h. The cells were then treated with 0.25 mg/mL RNase at 37°C for 15 min and the nuclei were stained by incubation in 50 µg/mL propidium iodide (PI) at 4°C for 30 min. DNA content (~20 000 events) were analyzed using a FACScan flow cytometry instrument equipped with FACStation running Cell Quest software (Becton Dickinson) for instrument control and data acquisition. The CV according to the FACStation Cell Quest soft-

Table 1. Summary with the number of probes which exhibited significantly altered levels of expression in HUVECs after exposure to the dp 3.9 oligomers fraction

Treatment	dp 3.9 *versus* DMSO (4 h)	Pre-cotreatment dp 3.9 45 min + TNF-α *versus* DMSO 45 min + TNF-α (6 h)
FDR cut-off value	<0.075	<0.075
Total number of selected probes	1318 (2.4%)[a)]	1036 (1.9%)
Total downregulated probes (ratio T/CT[b)] range, number of probes)	628 (47.6%)[c)]	572 (55.2%)
	(0.09 – 0.7, 356 probes)	(0.1 – 0.7, 352 probes)
	(0.7 – 0.96, 272 probes)[d)]	(0.7 – 0.95, 220 probes)
Total upregulated probes (ratio T/CT range, number of probes)	690 (52.4%)	464 (44.8%)
	(1.5 – 11.2, 221 probes)	(1.5 – 13.0, 258 probes)
	(1.05 – 1.5, 432 probes)[d)]	(1.07 – 1.5, 243 probes)

a) % of the total probes represented in the chip.
b) T: treated cells, CT: control cells.
c) % of the total selected probes.
d) Modest changes.

ware must be less than 10%. Data are presented as mean values ± SD of three independent experiments.

2.9 Cell migration: Wound healing assay

Confluent monolayers of HUVECs were scraped away horizontally with a sterile pipette tip. Media, dislodged cells, and debris were aspirated, washed once with PBS, and fresh full medium with or without test compounds was added back to the wells to initiate experiments. Randomly selected views along the scraped line were photographed on each well, using a phase contrast inverted microscope and a CCD camera attached to the microscope. Photographs were taken after 4, 24, and 48 h of incubation. The change in the area of the experimental condition was compared with that of the corresponding control. Results are representative of two independent experiments.

2.10 Statistical analysis

Where indicated, comparisons between mean values from control and treated samples were carried out with the two tailed unpaired Student's *t*-test. A p-value < 0.05 was considered significant.

3 Results

3.1 Analysis of differentially expressed genes

Analysis of gene expression data from the preliminary study in which cells were treated with two concentrations of EC or dimer B2, yielded only 29 and 20 changing probes that met the first of the two established selection criteria, respectively (Table S1 of Supporting Information). Among these probes and only in cells treated with EC there was one gene, *HINT3* (histidine triad nucleotide binding protein 3), that met both selection criteria and that therefore, was considered downregulated by the treatment. This gene encodes a protein that belongs to a superfamily of nucleotide hydrolases and transferases [21]. In a second set of experiments, where cells were treated with EC 25 μM (n = 3 independent experiments), it was found that 35 probes were upregulated (≥1.5) and 143 probes were downregulated (≤0.67). But for these probe changes the estimated FDR values were very high (>0.7, *i.e.*, >70% of false-positives predicted) (Table S2 of Supporting Information), and given the weak significance of the results, further cell treatments with EC or dimer B2 were not performed.

However, when cells were exposed to the mixture of oligomeric procyanidins, our analysis strategy detected above 1000 genes for which the expression levels were significantly altered both in resting and cytokine-stimulated cells (Tables S3 and S4 of Supporting Information, respectively). A summary of these results is presented in Table 1. We focused this research on those changes caused by the dp 3.9 fraction in HUVECs. The gene expression changes observed in resting cells exposed to the oligomeric compounds were compared to those obtained in TNF-stimulated cells (as a model of vascular dysfunction) also treated with the dp 3.9 procyanidins fraction. We observed that an important proportion of those changes induced by the procyanidins in the resting cells were similarly induced in the cytokine-stimulated cells (Table S5 of Supporting Information). We consider these findings important in that they represent a set of changes that are induced by the dp 3.9 oligomers in vascular endothelial cells regardless of the inflammatory status of the cells and confirmed that these changes were induced by the dp 3.9 oligomers fraction.

3.2 Functional analysis

Data containing the significantly regulated probes by dp 3.9 were uploaded into the Ingenuity software in order to query the Ingenuity Knowledge Database. This process generates

Table 2. Summary of the functional analysis performed with IPA and top regulated probes with significant altered levels of expression

IPA Results	dp 3.9 *versus* DMSO (4 h)	Preco-treatment dp 3.9 + TNF-α *versus* DMSO + TNF-α (6 h)	Common regulated probes by dp 3.9 alone or in the presence of TNF-α
Probes used in the analysis	1318	1036	278
Top bio functions			
Diseases and disorders	Cancer Haematological diseases	Inflammatory disease Cancer Haematological disease	Cancer CVDs Haematological disease Inflammatory disease
Molecular and cell functions	Cell death Cell growth and proliferation Cell movement Gene expression DNA replication, recombination, and repair Cell morphology	Cell growth and proliferation Cell death Cell movement Cell signaling Small molecule biochemistry	Cell death Cellular growth and proliferation Cell signaling Cellular movement Cellular development
Physiological system development and function	Cardiovascular system development and function Organismal, tissue, organ development	Immune response Tissue morphology Cardiovascular and haematological system development and function	Cardiovascular system development and function Organismal development Tissue morphology
Top upregulated genes	ADAMTS1 (+13.0), KLF4 (+11.0), HS3ST1 (+7.5), KITLG (+4.5), PPAP2B (+3.8)	RSAD2 (+11.2), IFIT1 (+8.5), LOC129607 (+6.7), OAS2 (+6.6), IFIT2 (+6.4)	ADAMTS1 (+13.0), KLF4 (+11.0), KITLG (+4.5), CCRL1 (+3.7), ARL4C (+3.5),
Top downregulated genes	BMP4 (−11.3), CTGF (-8.8), CXCR4 (−8.4), ET-1 (−8.3), CYR61 (−8.1)	ANGPT2 (−9.5), CTGF (−8.9), DKK1 (−8.4), APLN (−7.9), SPRY1 (−4.7)	BMP4 (−11.3), CTGF (−8.8), CXCR4 (−8.4), ET-1 (−8.3), CYR61 (−8.1)

significant biological networks and identifies genes that could be potentially involved in various cell functions and pathways. A summary of the top functions detected by this system is presented in Table 2. IPA functional analysis revealed that modulated genes were primarily associated with cancer, hematological, and CVDs as well as inflammatory diseases. The principal molecular and cell functions regulated by all the treatments were cell death, cell growth and proliferation, cell motility, cell signaling, gene expression, DNA replication and repair, cell morphology, and small molecule biochemistry. Additionally, the regulated genes were found to be involved in cardiovascular and hematological system development and function, organism, organ or tissue development as well as in immune response. The list of commonly regulated probes by dp 3.9 alone or in the presence of TNF-α (Table S5 of Supporting Information) was also uploaded to the Ingenuity software to further assess the cell functions in which these genes may be involved. It was confirmed that these genes were implicated in cancer, cardiovascular, hematological and inflammatory diseases and that, the main molecular cell functions influenced by the dp 3.9 fraction were cell death, cell growth and proliferation, cell movement, cell signaling, and cell development. Cardiovascular system development and tissue morphology emerged as the top physiological function in which the probes were implicated. The top regulated genes for each treatment are also shown in Table 2.

We further aimed at confirming the significance of the functional analysis by using the FatiScan tool. This system searches out for the biological functions in which the complete list of differentially expressed probes may be involved and allows the selection of significantly over-represented GO functional terms associated with genes most induced in treated cells *versus* control cells. Results are summarized in Table S6 of Supporting Information. When comparing unstimulated cells treated with the dp 3.9 fraction *versus* control, the following terms were found to be significantly over-expressed in the control cells: *cytoskeleton organization* and *biogenesis*, *cell differentiation*, *regulation of cell growth*, *morphogenesis of a tube*, and *blood vessel development* indicating that these functions were repressed in the cells treated with the dp 3.9 fraction. The term *cell division* was over-expressed in control cells *versus* the cytokine-treated cells co-treated with the dp 3.9 fraction indicative of an effect of the dp 3.9 fraction on cell division. Overall these results suggest that exposure of HUVECs to the oligomers fraction may have some effects on blood vessels formation and angiogenesis processes through modulation

Table 3. Relative changes in gene expression (ratio treated/control cells) of selected genes in HUVECs after exposure to the dp 3.9 oligomers fraction (in both resting and cytokine-stimulated cells), as determined by Affymetrix microarrays *versus* quantitative real time RT-PCR

Gene symbol	Function	dp 3.9 *versus* DMSO (4 h)			Preco-treatment dp 3.9 45 min + TNF-α *versus* DMSO 45 min + TNF-α (6 h)	
		Affyx	RT-PCR[a)]	RT-PCR[b)]	Affyx	RT-PCR[a)]
ADAMTS1	Inhibits endothelial cells proliferation.	13.0	14.26 ± 3.26[c)]	1.64 ± 0.33	3.1	3.07 ± 0.78
ANGPT2	Role in endothelial cells apoptosis, migration, proliferation	0.5	0.33 ± 0.03	0.35 ± 0.05	0.1	0.08 ± 0.01
CYR61	Can promote endothelial cell growth, migration, adhesion and survival *in vitro*	0.1	0.09 ± 0.01	0.20 ± 0.02	0.2	0.17 ± 0.02
EDG3	May contribute to the regulation of angiogenesis and vascular endothelial cell function	0.4	0.35 ± 0.05	0.43 ± 0.11	0.3	0.42 ± 0.10
ET-1	Responsible for endothelial cell proliferation, migration, invasion, and tubule formation.	0.1	0.06 ± 0.01	0.21 ± 0.02	0.2	0.22 ± 0.01
FLT1	Important for the control of cell proliferation and differentiation	2.1	1.32 ± 0.11	n.c.	1.5	n.c.
PDE4B	Inhibition of PDE4 activity decreased migration	0.5	0.43 ± 0.03	0.76 ± 0.07	0.6	0.58 ± 0.02
THBS1	Inhibitor of angiogenesis and of migration and proliferation of endothelial cells	0.3	0.17 ± 0.04	0.36 ± 0.04	0.2	0.24 ± 0.02

n.c.: No change (fold change = 1.0); RT-PCR.
a) Performed in the same RNA as the microarrays; RT-PCR.
b) Performed in RNA from an independent experiment.
c) Results represent mean value ±SD (n = 3).

of the expression of genes involved in cell growth, cell proliferation, and cell movement.

3.3 Validation of microarrays results by RT-PCR

We selected a subset of eight genes that showed significant altered levels of expression in response to the dp 3.9 extract exposure alone and in the presence of TNF-α and, which were associated by the IPA analysis to cardiovascular system and development functions, cell movement, cell growth and cell proliferation, and blood vessel morphogenesis (Table S7 of Supporting Information). For each gene, RT-PCR reactions were performed in aliquots of the same mRNA used for microarrays and in mRNA prepared from an independent experiment (each experiment consisted of triplicate mRNA samples extracted from three separate plates). The list of selected genes, their associated function and expression changes by microarrays and RT-PCR are presented in Table 3. The majority of the RT-PCR results are in agreement with the Affymetrix microarrays results. We were not able to confirm the induction of FLT1 transcripts.

3.4 Inhibition of ET-1 production by the dp 3.9 procyanidins

To confirm the observed changes at the protein level, we also measured the levels of ET-1 production and release to the cell culture media. After 4 h incubation of HUVECs with the dp 3.9 fraction alone, there was a significant decrease in ET-1 release to the culture medium (*$p < 0.05$; Fig. 2). The decrease in ET-1 production induced by the dp 3.9 fraction was also confirmed in cells co-treated with TNF-α, 10 ng/mL.

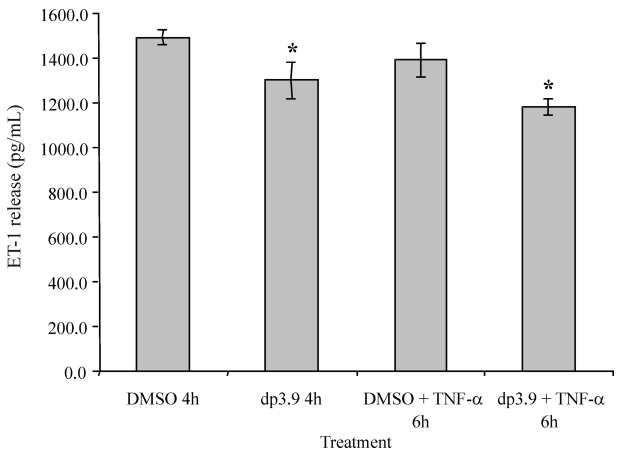

Figure 2. Release of ET-1 to the culture medium by HUVECs after exposure to the dp 3.9 oligomeric procyanidins fraction (10 μg/mL) alone for 4 h and in the presence of TNF-α (10 ng/mL) for 6 h. Results represent mean value ± SD ($n = 3$; *$p <$ 0.05).

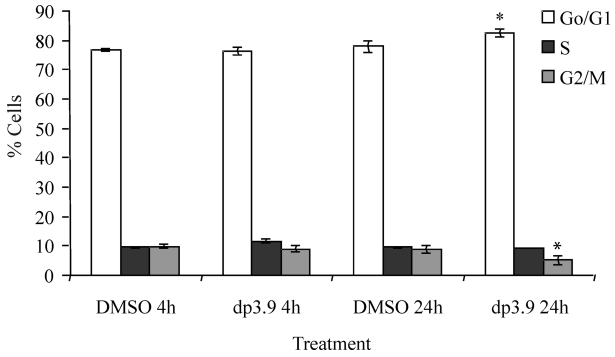

Figure 3. Distribution of HUVECs in the G_0/G_1, S, and G_2/M phases after exposure to the dp 3.9 oligomeric procyanidins fraction (10 μg/mL) for 4 and 24 h. The analyses of cell cycle distribution were performed in triplicate for each group of cells. Data are presented as mean values ± SD (significant differences between control and treated cells, $*p < 0.05$).

3.5 Phenotypic response of HUVECs to the dp 3.9 exposure

To determine whether the observed gene expression changes were associated with a phenotypic response of the cells, the effects of dp 3.9 on HUVECs were further evaluated by examining cell proliferation, cell cycle distribution, and cell migration for control, and dp 3.9 treated cells. After 24 h of exposure to the dp 3.9 fraction (10 μg/mL), cell proliferation, and viability were slightly inhibited by 11 and 7%, respectively. Regarding cell cycle, after 4 h of exposure to the dp 3.9 fraction, both control and treated cells, were primarily in the G_0/G_1 phase (76.9 ± 0.5% and 76.4 ± 1.3%, respectively), with a similar proportion of cells in the S phase (9.6 ± 0.2 and 11.7 ± 0.5, respectively) and in the G_2/M phase (9.9 ± 0.5 and 9.0 ± 0.8, respectively) (Fig. 3). After 24 h of incubation, cells treated with the dp 3.9 fraction exhibited a small although significant increase in the percentage of cells in the G_0/G_1 phase (82.4 ± 1.3%, $*p < 0.05$) concomitant with a decrease in the proportion of cells in the G_2/M phase (5.1 ± 1.4%, $*p < 0.05$). Endothelial cell migration is an important factor in angiogenesis and we therefore investigated whether the oligomeric procyanidins affected HUVECs migration using the wound healing assay (Fig. 4). The data show that, exposure of HUVECs to 10 μg/mL of the dp 3.9 fraction inhibits drastically cell migration in comparison to control cells after 24 and 48 h.

4 Discussion

Data presented here show that a mixture of oligomeric procyanidins purified from apple, with a mean dp of ~3.9, significantly modulated the expression levels of several genes that are associated with key angiogenesis-related functions,

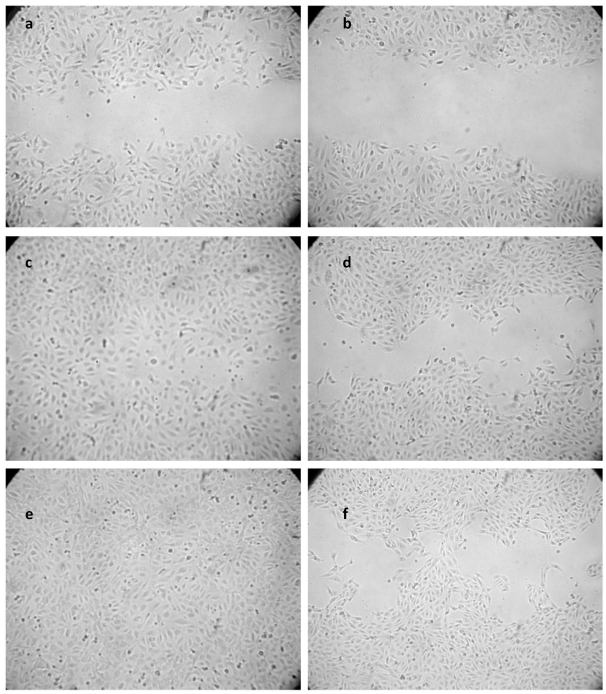

Figure 4. Effect of the dp 3.9 oligomeric procyanidins fraction on HUVECs migration examined by wound healing assay. Endothelial cell monolayers were wounded at time 0 and cultured with the dp 3.9 fraction (10 μg/mL). Photographs of control (a, c, e; DMSO alone, <0.1%) and treated cells (b, d, f) were taken after 4, 24, and 48 h of incubation, respectively. Results are representative of two independent experiments.

such as cell proliferation and cell migration, in HUVECs. These effects were not detected in cells exposed to EC or procyanidin dimer B2. In addition, treatment with dp 3.9 inhibited human vascular endothelial cell migration and proliferation and arrested the cells at the G_0/G_1 phase of the cell cycle.

Oligomeric procyanidins have been attributed with several cardioprotective effects including induction of eNOS and NO-mediated vasorelaxation in rat and human arteries [11, 22], and reducing blood pressure and improving cardiac performance in an experimental model of ischemia-reperfusion damage [23]. Further, it has been reported that trimers, tetramers, and pentamers are the most potent vasoactive polyphenols in red wine as shown by their ability to reduce ET-1 synthesis in endothelial cells [13] and that procyanidin tetramers and hexamers from cocoa are the most effective inhibitors of ACE [14]. Regarding effects on the angiogenic process, it has been reported that polyphenols from red wine and green tea are able to inhibit the proliferation and migration of endothelial cells and vascular smooth cells (VSMs), as well as the expression of major proangiogenic factors such as vascular endothelial growth factor (VEGF) and MMP-2 [10] but the identity of the components in these extracts that were responsible for the effects

have not been identified. Data reported by Kenny *et al*. [24] have shown that pentameric and octameric procyanidins from cocoa inhibit proliferation of endothelial cells and downregulate the expression of V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (ErbB2) tyrosine kinase, a receptor important in the regulation of angiogenesis. We specifically searched for expression changes (even if not picked up by our analysis) for probes representing *eNOS*, *ACE*, *VEGF*, *MMP-2*, or *ErbB2* but, in all cases, the ratios between treated and control cells for probes representing these genes were close to 1.0 and not significant.

Angiogenic function is progressively impaired with increasing age and impaired angiogenic function has been linked to the development and progression of a variety of pathological conditions including cancer and CVDs [25]. For example, the formation of new blood vessels has been observed in the atherosclerotic plaques of human coronary arteries and the number of new vessels was shown to correlate with the degree of inflammation and severity of the lesion [26]. Further, it has been reported that angiogenic inhibitors can suppress atherosclerotic plaque progression [27]. Angiogenesis is a very complex process regulated by a dynamic balance between positive and negative effectors. Growth factors such as VEGFs, fibroblast growth factors (FGFs) or transforming growth factors (TGFs), proteases such as MMPs or the urokinase-type plasminogen activator, cytokines/chemokines (IL-8, CXC) and other factors such as tissue factor (connective tissue growth factor (CTGF)) or prostaglandins synthase (PTGS) are examples of important angiogenic stimulating mediators whereas ANGPT2, THBS-1, proteases inhibitors, and platelet factor-4 are the inhibitors of angiogenesis [28]. Current efficient angiostatic agents such as endostatin exert a direct effect on migration, proliferation, and endothelial cell survival by altering the expression levels of several of these angiogenic factors [9]. Also, bioactive dietary polyphenols such as EGCG [29] has been shown to affect angiogenesis by modulating the levels of multiple angiogenic factors.

Data presented here show that exposure of vascular endothelial cells to oligomeric procyanidins causes significant changes in the transcript levels of a series of genes encoding proteins that have been associated with angiogenesis. For example, ADAMTS1, a secreted matrix metalloproteinase that has been shown to inhibit endothelial cell proliferation *in vitro* and to block growth factor-induced neovascular responses *in vivo* [30] was induced several-fold following treatment with the apple dp 3.9 fraction. The effects of ADAMTS1 appear to be mediated through the cleavage of THBS1 since the derived fragments have been shown to inhibit endothelial cell proliferation [31]. THBS1 is an extracellular glycoprotein with a role in vascular growth and morphogenesis and that suppresses migration and inhibits proliferation of endothelial cells [32]. The observed decrease in THBS1 transcript levels reported here may be associated with the inhibitory effects of apple oligomeric procyanidins on vascular endothelial cells. Other important angiogenic factors were also downregulated following exposure to the oligomeric procyanidins, including ANGPT2, a ligand for the tyrosine kinase Tie2 receptor that is expressed predominantly in endothelial cells. The ANGPT-Tie system is a key regulator of vascular maintenance and endothelial cells homeostasis [33]. Downregulation of ANGPT2 expression in HUVECs has been reported to be associated with the anti-angiogenic effects of some drugs [34]. Two members of the CNN family of proteins, CYR61 and CTGF were also downregulated in HUVECs by the dp 3.9 fraction. These extracellular matrix-associated signaling molecules have an important role in regulating endothelial cell function and can promote cell growth and migration [35]. We also show that genes encoding ET-1, a potent vasoactive peptide that promotes proliferation and migration in HUVECs [36], and the G-protein coupled receptor EDG3 that is known to enhance endothelial cell proliferation and migration [37] were downregulated in response to the dp 3.9 treatments. Another interesting protein that was also downregulated following exposure to the oligomeric procyanidins, was the phosphodiesterase 4B. PDE4B is an enzyme critical for the regulation of cAMP levels which plays an important role in modulating the migratory and proliferative tendencies of many cell types including vascular endothelial cells [38]. It has been reported that inhibition of PDE4B activity decreased migration in vascular endothelial cells and that inhibitors of this protein may represent novel therapeutic agents to limit angiogenesis in complex human diseases [39]. The changes in the transcript levels of *ADAMTS1*, *THBS1*, *ANGPT2*, *CYR61*, *EDG3*, *ET-1*, and *PDE4B* were all confirmed by RT-PCR. Other genes of interest that were strongly regulated in HUVECs after exposure to the dp 3.9 fraction both in resting and cytokine-stimulated cells were bone morphogenetic protein 4 (*BMP4*), apelin (*APLN*), chemokine (C-X-C motif) receptor 4 (*CXCR4*), KIT ligand (*KITLG*), and Kruppel-like factor 4 (gut) (*KLF4*). All these genes have also been shown to be involved in angiogenic processes and (or) vascular function and further research on their role in the migration inhibitory and regulatory properties of oligomeric procyanidins in human endothelial cells is warranted.

Oligomeric procyanidins are components of human diets and to exert their effects on the vascular endothelium they must be absorbed from the gastrointestinal tract. There are a number of reports concerned with the stability and bioavailability of procyanidins following ingestion but the metabolic fate of these compounds has not yet been completely clarified. In a study where the stomach contents of subjects were periodically sampled (*via* gastric intubation) following ingestion of a procyanidin-rich cocoa beverage, it was shown that the procyanidins were stable and there was no evidence of breakdown and release of flavan-3-ols either as monomers or oligomers [40]. These observations indicate

that the majority of ingested oligomeric procyanidins will enter the small intestine intact with the potential to be absorbed. A number of reports describe the appearance of procyanidins in plasma following oral ingestion. The absorption of procyanidin dimers and trimers has been demonstrated in rats and in humans, with reported plasma values in the range of 10 – 100 nM [18, 41]. A more recent report concerned with the absorption of procyanidins from an apple polyphenol extract and from purified oligomeric procyanidins of a single dp (dp range 2 – 6) that were fed to rats, detected procyanidins in the plasma at concentrations up to 10 μg/mL (equivalent to ~10 μM for a procyanidin tetramer) and with dp up to 5 [20]. Shoji *et al*. extracted the rat plasma samples after treating them with 8 M urea which facilitated unfolding of plasma proteins and prevented the typically strong protein binding that occurs with procyanidins; since classical direct solvent extraction techniques were used in the previously reported studies, it is possible that the plasma procyanidin concentrations in these were under-estimated. Our data show that at a concentration of ~10 μg/mL, the dp 3.9 apple procyanidin fraction caused significant changes in gene expression and inhibited migration of HUVECs.

In conclusion, the data presented here show that a mixture of oligomeric procyanidins, containing mostly trimers, tetramers, and pentamers, exerted migration-inhibitory effects in cultured human vascular endothelial cells. These effects were associated with changes in the expression of a series of genes encoding proteins that are involved in the angiogenic process. We propose that these molecular changes may be an important mechanism underlying the cardiovascular protection provided by oligomeric procyanidins and that some of the regulated proteins may be utilized as novel biomarkers for procyanidin exposure. Further work is required to validate whether changes in mRNA levels are translated into protein changes and to understand the molecular mechanisms underlying the cellular response. In addition, the regulatory and migration inhibitory properties of these compounds need to be explored in studies concerned with tumor progression.

We are very grateful to Dr. Jesus Cantalejo (Unidad de Genómica, Parque Científico de Madrid, Fac. Biología, Universidad Complutense, Madrid, Spain) for his help with the microarray analysis, Dr. Roy Bongaerts (Institute of Food Research, Norwich, UK) for advice and support with the flow cytometry and Amélie Casgrain (Institute of Food Research, Norwich, UK) for helping with the culturing of cells. The authors also thank D. Francisco L. Portillo for technical assistance with the HUVECs photographs. This research was supported financially by the Spanish MEC and FEDER (AGL2005-24849-E) and the European Commission FOOD-CT-2004-513960 (FLAVO).

The authors have declared no conflict of interest.

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