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ARTICLE

Regulation of Vascular Endothelial Function by Procyanidin-Rich Foods and Beverages†

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Flavonoid-rich diets are associated with a lower mortality from cardiovascular disease. This has been linked to improvements in endothelial function. However, the specific flavonoids, or biologically active metabolites, conferring these beneficial effects have yet to be fully defined. In this experimental study of the effect of flavonoids on endothelial function cultured endothelial cells have been used as a bioassay with endothelin-1 (ET-1) synthesis being measured an index of the response. Evaluation of the relative effects of extracts of cranberry juice compared to apple, cocoa, red wine, and green tea showed inhibition of ET-1 synthesis was dependent primarily on their oligomeric procyanidin content. Procyanidin-rich extracts of cranberry juice triggered morphological changes in endothelial cells with reorganization of the actin cytoskeleton and increased immunostaining for phosphotyrosine residues. These actions were independent of antioxidant activity. Comparison of the effects of apple procyanidin monomers through heptamer showed a clear structure−activity relationship. Although monomer, dimer, and trimer had little effect on ET-1 synthesis, procyanidin tetramer, pentamer, hexamer, and heptamer produced concentration-dependent decreases with IC_{50} values of 5.4, 1.6, 0.9, and 0.7 μM, respectively. Levels of ET-1 mRNA showed a similar pattern of decreases, which were inversely correlated with increased expression of Kruppel-like factor 2 (KLF2), a key endothelial transcription factor with a broad range of antiatherosclerotic actions including suppression of ET-1 synthesis. Future investigations of procyanidin-rich products should assess the role KLF2 induction plays in the beneficial vascular effects of high flavonoid consumption.

KEYWORDS: Flavonoids; endothelial function; endothelin-1; Kruppel-like factor 2

INTRODUCTION

The relationship between dietary factors and incidence of cardiovascular disease (CVD) is an important area of investigation for defining nutritional influences on optimal health. Lower mortality from CVD among red wine drinkers has been an important stimulus for these investigations (*1*). Although some reports attributed this simply to consumption of alcohol, comparison of the benefits of red wine with other alcoholic beverages led to the conclusion that additional components such as polyphenols (primarily flavonoids) contributed to these effects (*2*). In agreement with this, flavonoid-rich diets are also associated with a reduced risk of CVD (*3*, *4*).

Because endothelial dysfunction was recognized as an important precursor to CVD, including development of atherosclerotic lesions, the influence of flavonoids on vascular function and atheroma formation has been the focus of much of this research. Decreased endothelium-dependent vasodilatation, because of reduced nitric oxide output, and excess production of the vasoconstrictor endothelin-1 (ET-1) are key characteristics of endothelial dysfunction (*5*). In previous studies we identified oligomeric procyanidins (OPC) as the polyphenol component in red wine responsible for the inhibition of ET-1 synthesis by endothelial cells and concluded that OPC could account for the antiatherosclerotic actions of regular red wine consumption (*6*). Consistent with this, red wines from areas with a low incidence of heart disease (and increased longevity) had higher concentrations of OPC than wines from other areas (*6*). Interestingly, studies of dietary flavonoid intake in relation to reductions in risk of CVD show a stronger association for individuals consuming foods and beverages with high procyanidin content (*3*, *4*).

Daily consumption of cocoa or dark chocolate, which are also rich sources of procyanidins as well as other flavan-3-ols such as epicatechin, provides further support for the concept that a significant level of protection from CVD can be derived from daily consumption of products containing high amounts of OPC (*7*−*9*). Previous experimental studies of the effects of OPC

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on endothelium have used mainly sources of type-B procyanidins such as cocoa or grape seed extracts. Cranberry juice is another rich source of OPC. However, it contains high levels of both A-type and B-type procyanidins (*10*). B-type procyanidins are primarily composed of (−)-epicatechin subunits joined as oligomers linked through C4 of the heterocyclic ring and C8 in the flavonoid A-ring (*11*, *12*). A-type procyanidins are also epicatechin oligomers, but have a second linkage through an ether bond between C2 and C7 (*11*, *12*). Therefore, we investigated the relative inhibition of ET-1 synthesis by cultured endothelial cells using extracts of cranberry polyphenols and compared these responses to red wine, apple, and cocoa, which contain only B-type OPC.

Antioxidant actions of flavonoids and other polyphenols were thought to account for their health benefits (*12*). However, because of their poor bioavailability, it is now thought that their effects, including antiatherosclerotic properties, are due either to polyphenol metabolites or to specific highly potent molecules with selective physiological actions, which can be evoked by circulating amounts at nanomolar concentrations (*12*). The mechanism accounting for the vascular actions and beneficial cardiovascular effects of polyphenols remains to be elucidated. Although screening for biological activity using extracts on cultured cells or isolated tissues may not be the ideal model for comparison with dietary consumption of these products, it can provide insights into the molecules of greater potential impact on health and so assist the design of dietary intervention studies. In addition, cell studies may shed light on particular mechanisms to investigate or biomarker responses to evaluate.

Many studies have focused solely on improved endothelium-dependent vasodilator responses mediated by increased nitric oxide output. However, observations that OPC-rich extracts not only evoked endothelium-dependent vasodilatation but also suppressed ET-1 synthesis suggested a wider spectrum of actions. When considered in the context of morphological changes induced in cultured endothelial cells by OPC, we noted the close parallel between these responses and those induced by laminar shear stress. This led us to hypothesize that OPC triggered a laminar shear stress like response in the endothelium (*5*). Investigations over the past few years into the mechanisms regulating the laminar shear stress response in endothelial cells have demonstrated that the majority of changes in gene expression are governed by the transcription factor, Kruppel-like factor 2 (KLF2) (*13*), which acts as a master switch in the endothelium. KLF2 suppresses pro-inflammatory and pro-atherosclerotic genes such as ET-1 and induces antithrombotic and thrombolytic genes (*13*).

Here we describe the actions of cranberry extracts on ET-1 synthesis compared with red wine, apple, and cocoa extracts. We have also investigated morphological changes induced by fractionated cranberry extracts in endothelial cells and concluded that these responses are not due to antioxidant properties. Further insights into the structure−activity relationship of the effects of OPC on endothelial cells have been obtained from comparison of the actions of apple OPC of different sizes on ET-1 synthesis and the relationship with KLF2 expression.

MATERIALS AND METHODS

Reagents. All cranberry products (raw and pasteurized cranberry juice, 25% cranberry juice drinks (light and sugar-sweetened), and cranberry extract) were from Ocean Spray Cranberries, Inc., Lakeville, MA. Cocoa polyphenol extract was from Barry Callebaut, Wieze, Belgium. Green tea extract was prepared from commercially available green tea by maceration 20% (w/v) in a solution of 20% (v/v) ethanol for 2 h at room temperature. The apple extract and purified apple procyanidins were prepared from unripe apples (*Malus pumila* cv. Fuji) as previously described (*14*). (−)-Epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), and all other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Preparation of Samples for Cell Studies. Polyphenol extracts from cranberry juice and red wine were prepared using C18 Sep-Pak cartridges (Waters, Milford, MA) or Sephadex LH-20 as described (*2*, *6*). For fractionation of samples on Sephadex LH-20 (*15*) minicolumns were used (6 × 10 mm). Unbound components were rinsed from the column with 2 mL of 0.2% formic acid containing 30% methanol. Low molecular weight polyphenols were eluted with 3 mL of 60% methanol containing 0.2% formic acid. OPC were eluted with 2 mL of 70% acetone containing 0.2% formic acid (*6*). Eluted samples were dried at room temperature under a stream of N_2. Extracts were reconstituted in aqueous solution and diluted in culture medium for cell studies. Polyphenol content of samples was assayed using Folin−Ciocalteu reagent with catechin as a reference and expressed as catechin equivalents (CE). To reduce interference from nonphenolic components, polyphenol measurements were made on samples after extraction on C18 Sep-Pak or Sephadex LH-20.

Cell Studies. Bovine aortic endothelial cells (BAEC) were grown in 24-well plates with Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum as previously described and used when confluent (*2*, *5*, *6*). To study effects on ET-1 synthesis, polyphenol extracts were incubated with BAEC for 6 h at 37 °C in a humidified incubator with an atmosphere of 5% CO_2 in air. Conditioned medium samples were collected and subjected to ET-1 immunoassay by double-recognition site sandwich ELISA using ET-1 specific capture and detection antibodies (*16*). The absence of cytotoxic effects on BAEC was confirmed by measuring mitochondrial dehydrogenase activity with methylthiazoletetrazolium (MTT) at the end of the incubation period.

Immunocytochemistry. BAEC were cultured on glass coverslips and treated for 30 min at 37 °C with DMEM alone or with cranberry juice fractions from Sephadex LH-20 diluted in DMEM. Cells were then fixed for 30 min with 4% paraformaldehyde and permeabilized for 10 min with 0.5% Triton X-100 for dual-label immunofluorescence. Phosphotyrosine (PY)-containing proteins were detected with anti-PY monoclonal antibody 4G10 and rhodamine red-X-conjugated donkey anti-mouse IgG secondary antibody. F-actin was localized with FITC-conjugated phalloidin. Coverslips were mounted and photographed using a Zeiss fluorescence microscope with digital camera.

RNA Extraction and qRT-PCR Assessment of Cell Responses. Total RNA was extracted using a StrataPrep Total RNA Miniprep Kit (Stratagene). Reverse transcription was performed using the reverse transcription system (Promega). PCR (40 cycles) was performed with SYBR Green PCR Master Mix (ABgene) or by Taqman-based methodology on an Applied Biosystem 5700 detection system as previously described (*17*, *18*). Bovine RNA polymerase 2 (*POLR2A*) was used as a reference gene to normalize the levels of mRNA for the test genes (*19*). Primers were supplied by Eurogentec. The sequence accession numbers, forward and reverse primers were as follows: *KLF2*, XM_599191 (predicted), forward, AGCCCTACCACTGCAACTGG, reverse, GGTAGTGGCGCGTGAGCT; *EDN1* (preproET-1) S37093 (mRNA), forward, AAGAGTGTGTCTACTTCTGCCATCTG, reverse, AAAGAAGTCCTTTAAGGAGCGCT, probe, FAM-TGGGTCAACACTCCAGAGCACGTTGTT-BHQ; and *POLR2A*, XM_596010 (predicted), forward, CGATTAAGAAGGCCAAGCAGG, reverse, TCGTTGAGAATGCGGTTCAC.

Data Analyses. Immunoassay values and mRNA levels were expressed relative to values for untreated cells and reported as means ± sem. Results were analyzed using Graphpad Prism (GraphPad Software, San Diego, CA), and by ANOVA using Statview (SAS Institute Inc., Cary, NC) with Fisher's least significant difference as a post hoc to determine significance. The IC_{50} value is the concentration of test substance reducing basal ET-1 secretion by 50%.

RESULTS

Cranberry juice caused a concentration-dependent inhibition of ET-1 synthesis with an IC_{50} value equivalent to ≈2 μL/mL. This was comparable to the median value for 165 red wines studied previously (median, 25th and 75th percentiles: 2.0, 1.5,

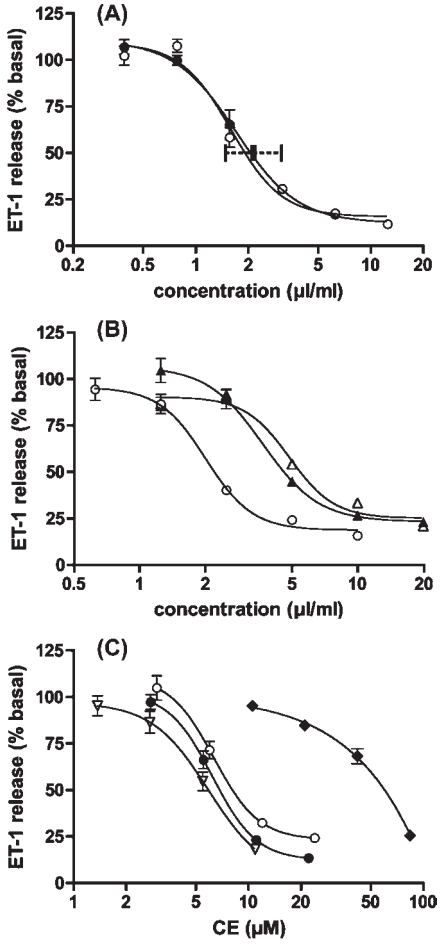

Figure 1. Inhibition of ET-1 synthesis by cranberry juice polyphenols. (**A**) Effect of raw (○) and pasteurized (●) cranberry juice; median IC_{50} value for red wines tested in this bioassay (*6*) (n = 165) with 25th and 75th percentiles indicated. (**B**) Representative Cabernet Sauvignon red wine (○), 25% cranberry juice drinks (light (▲) and sugar-sweetened (△)). (**C**) Comparison of the effect of polyphenol extracts from cranberry (○) with apple (●), cocoa (▽), and green tea (◆) expressed as catechin equivalents (CE). Data are from two or three experiments performed in triplicate.

and 3.1 μL/mL, respectively) (*6*) (**Figure 1A**). Pasteurization did not alter the biological effect of cranberry juice. Comparison of cranberry juice drinks (containing 25% cranberry juice) with a representative Cabernet Sauvignon red wine showed a lower degree of inhibition of ET-1 synthesis consistent with the lower levels of procyanidins in these products (**Figure 1B**). Expressed relative to polyphenol content cranberry, apple and cocoa extracts suppressed ET-1 synthesis with similar potencies. These extracts showed an approximately 10-fold greater potency than green tea extract (**Figure 1C**). The most likely explanation for this difference is that green tea extracts are composed mainly of flavan-3-ol monomers, whereas cranberry, apple, and cocoa polyphenol extracts all contain a significant proportion of OPC.

Fractionation of cranberry extracts on Sephadex LH-20 showed the greatest degree of inhibition of ET-1 synthesis was observed with the fraction eluted with 70% acetone (**Figure 2A**). Previous studies and characterization of apple procyanidin elution from Sephadex LH-20 (**Figure 2B**) show that elution with 60% methanol yields a fraction that is mainly composed of monomers, dimers, and trimers, whereas the 70% acetone eluant has the highest proportion of pentamers and larger OPC (*15*).

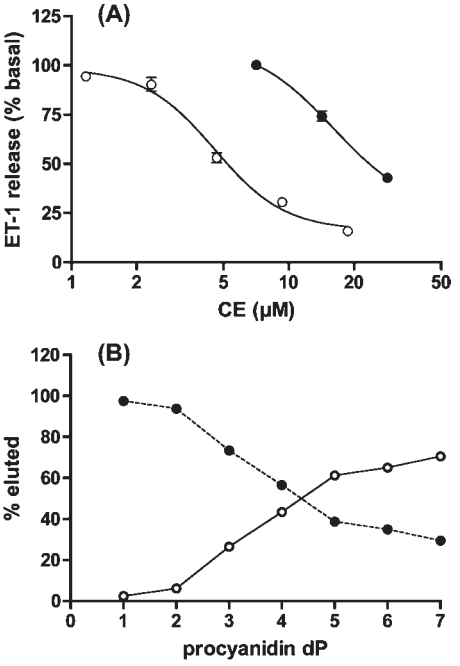

Figure 2. Fractionation of cranberry juice polyphenols on Sephadex LH-20. (**A**) Comparison of inhibition of ET-1 synthesis by polyphenols eluted from Sephadex LH-20 with 60% methanol (●) and 70% acetone (○). Data are from two experiments performed in triplicate. (**B**) Characterization of the elution characteristics of Sephadex LH-20 for apple procyanidins with different degrees of polymerization (dP) on sequential elution with 60% methanol (●) and 70% acetone (○).

These Sephadex LH-20 fractions were tested on cultured BAEC for their ability to induce morphological changes including localization of PY-containing proteins to points of cell−cell contact and reorganization of the actin cytoskeleton. Untreated cells (**Figure 3A**,**B**) showed only low levels of PY-containing proteins or F-actin. In comparison, the 70% acetone fraction induced marked changes in both the level of PY-containing proteins and F-actin and a striking colocalization at points of cell−cell contact (**Figure 3C**−**F**). Although the test concentrations of the methanol-eluted fraction from Sephadex LH-20 used for incubations shown in **Figure 3G**,**H** were greater, in terms of both polyphenol and antioxidant activity, than in **Figure 3E**,**F**, morphological changes did not occur. On the basis of these observations and the lower level of suppression of ET-1 synthesis by this fraction, it is unlikely that effects on the endothelium from OPC are due to antioxidant actions.

Further evidence that these effects on endothelial cells are dependent on a specific structure−activity relationship and independent of antioxidant activity is provided in **Figure 4**. EGCG caused a concentration-dependent inhibition of ET-1 synthesis, whereas ECG had no effect (**Figure 4A**,**B**) even though it is reported to have antioxidant capacity equivalent to EGCG (*20*). Observations that ECG and other monomeric flavan-3-ols at comparable concentrations have no effect on ET-1 synthesis (*21*) indicate that antioxidant activity is not involved in these endothelial responses. In contrast, evaluation of the relative inhibition of ET-1 synthesis with apple procyanidin monomer through heptamer revealed a clear structure−activity relationship (**Figure 4C**). Monomer, dimer, and trimer had little effect on ET-1 synthesis at the test concentrations. Procyanidin tetramer, pentamer, hexamer, and heptamer produced concentration-dependent decreases with the following IC_{50} values (μM, with 95% confidence limits): 5.4 (2.6−11.2), 1.6 (1.2−2.0), 0.9 (0.8−1.1), and 0.7 (0.6−0.8), respectively (**Figure 4C**).

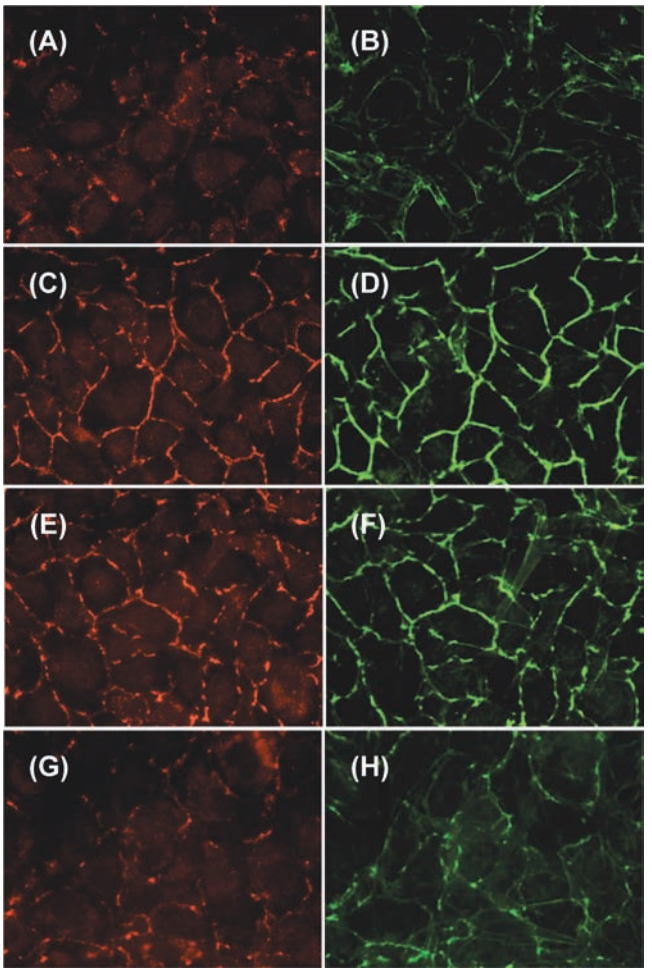

Figure 3. Morphological changes induced in cultured endothelial cells by cranberry juice polyphenols after fractionation on Sephadex LH-20 assessed by dual label immunofluorescence: (**A**, **C**, **E**, and **G**) phosphotyrosine immunofluorescence; (**B**, **D**, **F**, and **H**) actin cytoskeleton. (**A**/**B**) vehicle-treated cells; 70% acetone fraction (**C**/**D**) 9 μM CE, (**E**/**F**) 4.5 μM CE; and 60% methanol fraction, 7 μM CE (**G**/**H**). Magnification $\times$40.

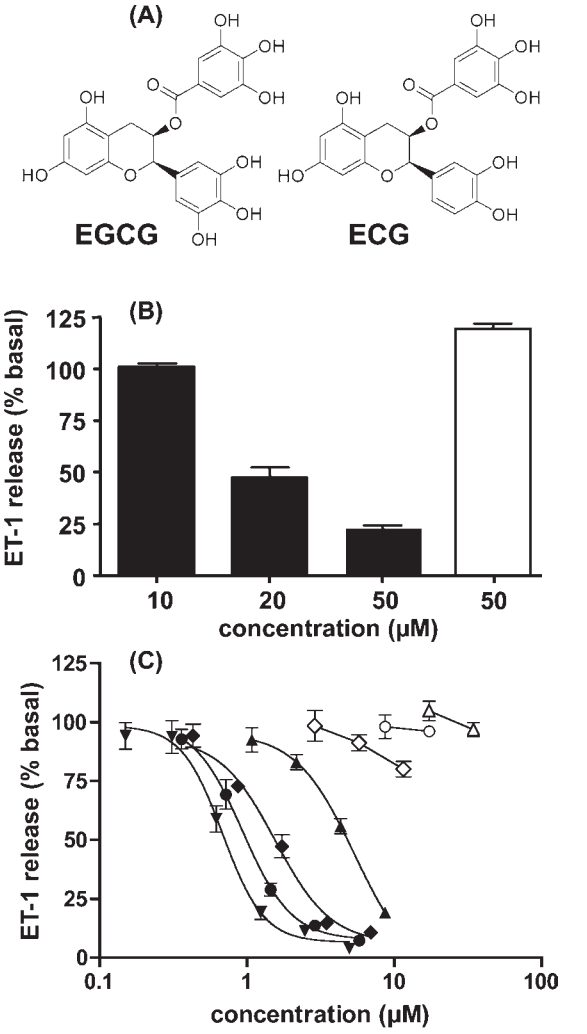

Figure 4. Structure−activity relationship for flavan-3-ol inhibition of ET-1 synthesis: (**A**) (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG); (**B**) EGCG inhibition of ET-1 synthesis (solid columns), compared with ECG; (**C**) relative inhibition with apple procyanidins (monomer (△), dimer (○), trimer (◇), tetramer (▲), pentamer (◆), hexamer (●), and heptamer (▼)). Data are from two or three experiments performed in triplicate.

The effects of apple procyanidin trimer, tetramer, and pentamer on levels of KLF2 and ET-1 mRNA are shown in **Figure 5**. Compared to results in **Figure 4C**, changes in levels of ET-1 mRNA showed a similar pattern with trimer having no effect, tetramer causing decreases only at the highest concentration (5 μg/mL, 4.3 μM), and pentamer causing concentration-dependent suppression of ET-1 at 2.5 and 5 μg/mL (1.7 and 3.5 μM) (**Figure 5B**). Importantly, given that KLF2 exerts an inhibitory regulation of ET-1 gene expression (*13*), increases in KLF2 mRNA were inversely correlated with ET-1 mRNA levels (r^2 = 0.78), supporting the concept that these changes are linked.

DISCUSSION

Cranberry consumption is mostly studied in relation to the beneficial effects of A-type procyanidins on urinary tract health (*10*, *11*). However, when the antiatherosclerotic actions of OPC are also considered, the daily consumption of cranberry juice is likely to have multiple health benefits.

In our earlier studies of red wine we observed that inhibition of ET-1 synthesis by OPC was most marked in the tetramer to pentamer range, whereas monomers (epicatechin and catechin) lacked this activity (*6*, *21*). Studies of endothelium-dependent vasodilatation using OPC isolated from grape seed extract or cocoa have also shown monomers and dimers lack activity (*22*, *23*). Again, tetramers and pentamers, as well as larger oligomers, were responsible for the endothelium-dependent vasodilator effects (*22*, *23*). This indicates a common mechanism of action for stimulating nitric oxide release and inhibiting ET-1 synthesis. More recently, microarray studies of cultured human umbilical vein endothelial cells (HUVEC) have shown an apple OPC fraction with a mean degree of polymerization (dP) of 3.9 caused a wide range of significant changes in gene expression, but no changes were observed in cells exposed to epicatechin or procyanidin dimer B2 (*24*). The latter study also reported inhibition of ET-1 synthesis with the apple dP 3.9 extract, but compared to the OPC response obtained on bovine or human aortic endothelial cells here and in previous studies (*2*, *5*), the response of HUVEC is modest (*24*).

The results described here show that cranberry juice has a profile of actions on the endothelium very similar to those of red wine and other OPC-rich foods and drinks. Purification and characterization of A-type procyanidins from cranberry extracts is needed to understand whether their profile of activity is similar to those reported here and previously for B-type procyanidins. Moreover, comparative analyses of the bioavailability of A-type

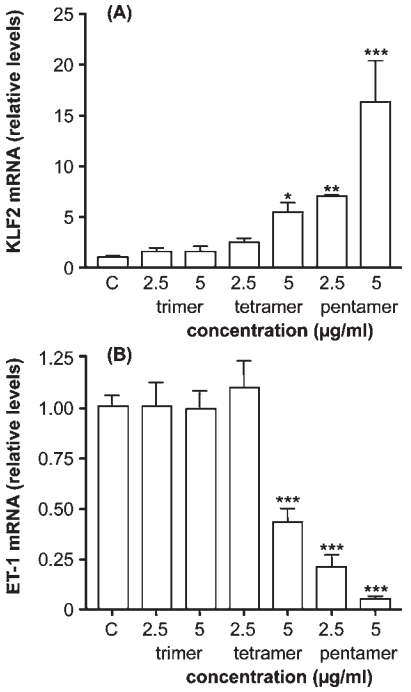

Figure 5. Relative mRNA levels for KLF2 (**A**) and ET-1 (**B**) after 1 h of stimulation of endothelial cells with procyanidin trimer, tetramer, and pentamer. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$. Data are from two experiments performed in duplicate.

and B-type procyanidins also need to be investigated in relation to changes in endothelial function. This is a critical consideration to correlate with these in vitro actions on vascular endothelial cells. Further evaluation of the minimum concentration of OPC eliciting an endothelial response is required because only effects occurring at nanomolar concentrations are thought to have a physiological relevance (*12*). However, such conclusions are based on measurements of venous blood, rather than samples of arterial blood in which local concentrations are more relevant for studying endothelial function in resistance arteries. Here, low micromolar concentrations of pentamer to heptamer were required to inhibit ET-1 synthesis. In the context of a perfused vessel, where there may be local accumulation of OPC, the threshold concentration may in fact be much lower, which may account for the discrepancy between isolated systems and in vivo responses.

Immunocytochemical investigations of responses led us to conclude that changes in cytoskeleton and tyrosine kinase signaling triggered by OPC treatment had a close similarity with the effect of laminar shear stress on endothelial cells (*5*). This was an important observation as laminar shear stress plays a key protective role in the vasculature by inducing a healthy endothelial phenotype, characterized by resistance to atheroma formation, promoting vasodilatation, and conferring an antithrombotic state. Atheroma most commonly occurs in the vascular tree at sites of nonlaminar shear stress or turbulent flow, notably at arterial bifurcations. Hence, if OPC can induce in these areas at risk of atheroma a comparable response to laminar shear stress, then these dietary components will produce an atheroma-resistant state when these protective physical forces generated by blood flow are absent (*5*). Consistent with this concept, low-dose red wine consumption reduces perturbed shear stress induced atherogenesis (*25*). In support of the hypothesis that OPC trigger a pseudo-laminar shear stress response in endothelial cells, we now report that OPC increase KLF2 expression. Increased KLF2 expression was also noted in the list of gene changes recorded in the microarray study of the effects of apple dP 3.9 on HUVEC (*24*). These findings add further support to the notion that OPC trigger a wide profile of changes in endothelial function by activating intracellular mechanisms identical to those mediating the response to laminar shear stress.

KLF2 has multiple actions. Without exception, these appear to confer a beneficial phenotype on the endothelium, making it resistant to changes generally associated with endothelial dysfunction and atherosclerosis (*13*). The profile of OPC initiating this KLF2 response has an identical pattern to that seen in experimental studies of endothelium-dependent vasodilatation and inhibition of ET-1 synthesis (*6*, *22*, *23*). Interestingly, it has been suggested that strategies to increase KLF2 expression could be therapeutically useful for prevention of atherosclerosis (*26*). The results described here indicate that this could be achieved through OPC administration or, indeed, with synthetic analogues with improved bioavailability. Observations of a structure−activity relationship in terms of characteristics of active OPC molecules, and downstream signaling mechanisms leading to changes in gene expression, are consistent with a receptor-mediated response (*27*). However, the precise target protein has yet to be identified. Future investigations are likely to provide insights into the biology of dietary OPC consumption and may identify pharmaceutical strategies with a similar or greater benefit.

In agreement with previous studies on cultured endothelial cells or isolated vessels, compared to flavonoid monomers, OPC have by far the most potent effects on endothelial function. Whether this is also true following dietary consumption of products containing OPC requires a more detailed examination of the relationship between bioavailability and functional effects. Identification of the role played by KLF2 in the response to dietary procyanidins increases the scope to identify biomarkers of this response that can be used to assess optimal consumption patterns of OPC-rich foods and beverages and to determine factors affecting bioavailability and metabolism of this family of polyphenols.

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