Cocoa Procyanidins Inhibit Proliferation and Angiogenic Signals in Human Dermal Microvascular Endothelial Cells Following Stimulation by Low-Level H_2O_2

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Procyanidins extracted from cocoa play a role in the defense against oxidative stress, as well as in vascular and immune functions. We previously reported that pentameric procyanidins isolated from cocoa inhibit the expression of the tyrosine kinase ErbB2 gene, thus slowing the growth of cultured human aortic endothelial cells. We herein investigate the further consequences of such inhibition by cocoa procyanidins, particularly regarding the protein level in phosphorylation patterns and the effects on the proliferation of human dermal microvascular endothelial cells (HDMECs) following angiogenic stimulation with low-level H_2O_2. We report herein that both the pentameric and octameric procyanidin fractions of cocoa inhibit the proliferation of HDMECs, whereas the pentameric fraction modulates the activity of several crucial proteins in angiogenic signaling by altering their tyrosine phosphorylation. Similar to aortic endothelial cells, the pentameric procyanidin fraction down-regulates the expression of ErbB2 tyrosine kinase in HDMECs. In conclusion, we report evidence suggesting that polyphenols may influence endothelial growth signaling, thus affecting angiogenesis *in vitro*. If these observations are applicable *in vivo*, they suggest a beneficial effect for cells overexpressing ErbB2, such as in specific neoplasias. Exp Biol Med 229:765–771, 2004.

Key words: cocoa; angiogenesis; procyanidins

Epidemiological and metabolic data indicate that the dietary consumption of fruits and vegetables containing flavanols and procyanidins reduces the risk of developing certain chronic diseases associated with oxidative stress, including specific forms of vasculopathy (1, 2). Among such aliments, seeds from the fruit of *Theobroma cacao* also contain variable amounts of flavanols and procyanidins (flavanol oligomers) with a wide variety of chemical structures and isomers; these molecules may exert a protective effect against human diseases (3–5). In particular, cocoa flavanols and procyanidins influence cellular defense mechanisms against oxidative stress, as well as the vascular and immune functions (6–14). Oxidative stress can disrupt a number of cellular pathways and trigger cell apoptosis through the activation of the stress-activated protein kinase and nuclear factor-κB pathways (15). Several reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), interact with cell proteins and lipids, thus intervening in a number of physiological cell mechanisms (16). Accordingly, low amounts of ROS are regarded as cofactors in the regulation of many intracellular signaling cascades, including those determining angiogenesis (17). Moreover, the addition of ROS to cultured cells activate membrane receptor tyrosine kinases, including platelet-derived growth factor receptor (18), insulin receptor (19), and ErbB kinases (20, 21). Similarly, ROS can stimulate the p21 Ras-MAPK (i.e., mitogen-activated protein kinase) pathway; this activity links the membrane receptors with the nucleus (22). A rise in levels of endogenous H_2O_2 can be achieved via the addition of several growth factors to cells (23), thus suggesting an autocrine or paracrine effect (or both) of ROS in cell proliferation such as angiogenesis. The specific molecular targets of cocoa flavanols and procyanidins within the H_2O_2-mediated proliferation are unknown but would be influenced by cell types, environmental factors, differentiation stages, or a combination of these.

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We herein investigated the cellular and molecular activity of cocoa flavanols and procyanidins (in their dimeric, pentameric, and octameric isoforms) in human dermal microvascular endothelial cells (HDMECs) and the changes in such activity following stimulation with H_2O_2. First, we observed that the pentameric procyanidin fraction isolated from cocoa is a potent modulator of tyrosine kinase expression. Further, we observed that the pentameric procyanidin fraction down-regulates the expression of the ErbB2 receptor tyrosine kinase. Moreover, following treatment with the pentameric procyanidin fraction, endothelial cells had lower protein tyrosine kinase phosphorylation on other proteins involved in angiogenesis regulation. Finally, both the pentameric and octameric procyanidin cocoa fractions suppressed the growth of HDMECs. The data reported herein confirm our previous data in a different cell model (24) while significantly extending our knowledge of the effects of procyanidins on angiogenesis.

Materials and Methods

Purification of Cocoa Flavanols and Procyanidins by Preparative Normal-Phase High-Performance Liquid Chromatography. Purification of the flavanol and procyanidin fractions from cocoa was conducted as described previously (10). Separations were performed using a 5-m Supelcosil LC-Si 10.0 nm column (Supelco, Bellefonte, PA). Purity was greater than 93% for the flavanol and pentameric procyanidin fractions, with the impurities being tetrameric and hexameric procyanidins in the pentameric fraction. The octameric procyanidin fraction purity was greater than 95%, with impurities primarily comprised of heptamers and monomers.

Culture Conditions. HDMECs, attachment factor solution, and CADMEC growth media were obtained from Cell Applications (San Diego, CA). Cells were cultured in T25 flasks (coated with attachment factor according to the manufacturer's instructions) and were incubated at 37°C in 5% CO_2. At 80% confluence, cells were subsequently subcultured into new T75 flasks. All experiments were conducted using cells with a low passage number. HDMECs were trypsinized and removed from the T75 flasks and plated into two 6-welled plates (coated with attachment factor). Cells in each plate were treated for 48 hrs with varying concentrations of cocoa flavanols, pentameric and octameric procyanidins, and controls. One-micromolar H_2O_2 in Hanks balanced salt solution was added for 30 mins for angiogenic stimulation. The H_2O_2/media was removed and replaced with fresh CADMEC growth media. After 1 hr, treated and control cells were lysed on ice with either 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) or 2 ml of lysis buffer (25 m*M* Hepes pH 7.6, 0.3 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.1% Triton X-100, 0.5 m*M* dithiothreitol [DTT], 0.1 m*M* sodium orthovandate, 20 μg/ml leupeptin, 1 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml Aprotinin, 10 μg/ml Pepstatin, and 1 m*M* NaF). Cell lysates were aliquoted and stored at −80°C.

RNA Isolation. For RNA isolation, 10^6 treated and nontreated HDMECs were lysed with 1.0 ml TRIzol reagent (Invitrogen). The RNA was isolated and frozen at −80°C. Five micrograms of total RNA was used for reverse transcription using Superscript II RT (Invitrogen) and 1.0 μl of cDNA was used for polymerase chain reaction (PCR).

Kinase Profiling. The kinase profiling assay was carried out as previously described (25) with slight modifications. Essentially, various tyrosine kinase transcripts were amplified with degenerate primers. The PCR annealing temperatures were initially at 45°C for 5 cycles followed by 55°C for the final 25 cycles. The PCR-amplified products from HDMEC samples were electrophoresed in a 2.4% agarose gel (3:1 ratio of Nusieve GTG:agarose LE; BMA, Rockland, ME). The 153–177 base pair bands were excised from the gel and DNA was purified using the QIAEX II gel extraction kit (Qiagen, Valencia, CA). Equivalent amounts of radioactive DNA from each sample were digested with restriction enzymes (New England Biolabs, Beverly, MA), after which the digested products were resolved on a 7% acrylamide gel (10:1 acrylamide:bisacrylamide; Bio-Rad Laboratories, Hercules, CA). The gel was then dried and subjected to autoradiography.

Quantitative Real-Time PCR. Quantitative real-time PCR was performed using a GenAmp 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Briefly, optimal PCR primers are designed for each kinase using Primer Express software (PE Biosystems) to specifically amplify the gene of interest. The primer sequences for ErbB2 are as follows: forward, 5′-AGGGAAAACACATCCCCCAA; reverse, 5′-TTGGCAATCTGCATACACCAG. For KDR, the primers are as follows: forward, 5′-CTTCCAAGTGGCTAAGGGCA; reverse, 5′-GGCGAGCATCTCCTTTTCTG. For Mapk 11 the primer sequences are forward, 5′-ACGCCCGGACATATATCCAG; reverse, 5′-GTCCAGCACCAGCATCCTTC. Primers for the housekeeping gene GAPDH were purchased from PE (Perkin Elmer, Boston, MA). PCR was performed using SYBR Green technology. Standard curves were prepared for both target and housekeeping genes from pre-prepared reference samples. The relative concentrations of the target gene were determined from the standard curve and normalized to the amount of housekeeping gene for each sample.

Proliferation. To assess cell proliferation, HDMECs in 1.0 ml (2.5×10^4 cells/15-mm wells) were cultured on fibronectin-coated wells with the Human Endothelial–Serum-Free Medium System (Invitrogen) supplemented with epidermal growth factor (EGF) and β fibroblast growth factor (βFGF; 10 ng/ml) in the absence or presence of vascular endothelial growth factor (VEGF; 10 ng/ml) as indicated. Triplicate wells of endothelial cells were treated (10–50 μg/ml) with cocoa flavanols and pentameric and

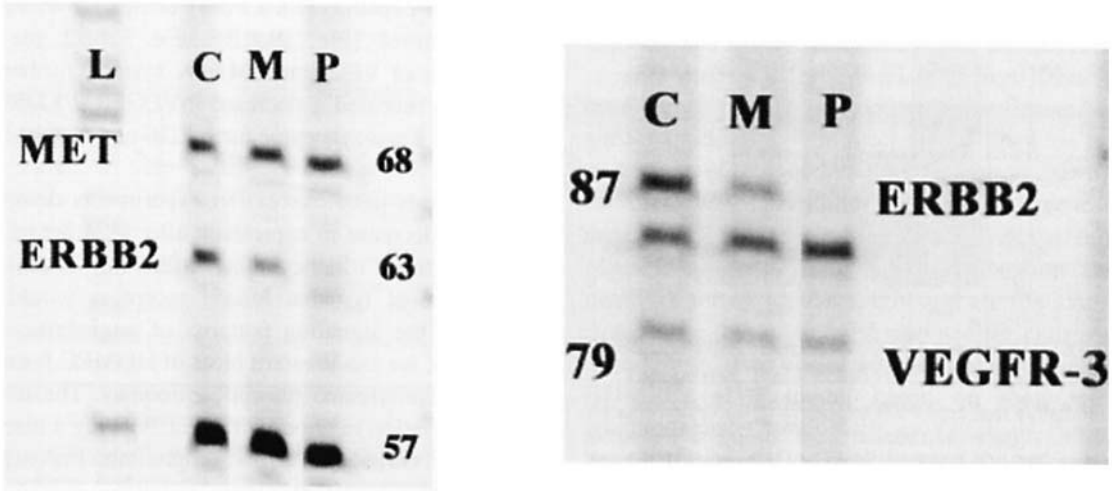

Figure 1. (Left panel) Autoradiograph of a kinase profiling polyacrylamide gel electrophoresis displays the restriction digest of *Rsa*I of tyrosine kinase PCR products of HDMECs treated with the various cocoa preparations (L, ladder; C, control saline; M, monomer; P, pentamer). Digest bands representing tyrosine kinase MET and ErbB2 have been identified. ErbB2 is down-regulated by pentameric treatment, whereas the kinase MET is unaffected. (Right panel) Autoradiograph of a kinase profiling PAGE gel displays the restriction digest of Bsp1286 of tyrosine kinase PCR products of HDMECs treated with the various cocoa preparations (C, control saline; M, monomer; P, pentamer). Digest bands representing tyrosine kinase ErbB2 and VEGFR-3 have been identified. ErbB2 is again down-regulated by the pentameric treatment similar the *Rsa*I digestion.

octameric procyanidins, or untreated (controls) for 48 hrs. Cells were treated with H_2O_2 as described above, and cultured for 36 hrs. Then, cells were pulsed with 0.6 μCi (26) of thymidine/15-mm well for 12 hrs and (26) thymidine incorporation into DNA was measured as an index of cell proliferation (26).

Western Blotting and Immunoprecipitation. Tyrosine-phosphorylated proteins were detected by immunoblotting. Samples of control and treated lysates (20 mins after H_2O_2 treatment) were thawed and kept on ice. Twenty micrograms of each sample were boiled with sample loading buffer for 2 mins. The gels were run at 25 mA for 1 hr, transferred to a nitrocellulose membrane, and blocked with 1% bovine serum albumin. Blots were probed with optimally diluted mouse monoclonal RC2OH-HRP (Transduction Laboratories, Lexington, KY) and mouse monoclonal antibody PY-20 (Zymed, South San Francisco, CA). After 1 hr, the blots were washed four times with 10 m*M* Tris, 100 m*M* NaCl-Tween-20, and incubated with 10^{-4} dilution of horseradish peroxidase–conjugated goat anti-mouse immunoglobulins (Zymed). Proteins were visualized with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). All blots were repeated a minimum of three times.

Results

The endothelial cells involved in tumor angiogenesis, wound healing, and inflammation are predominantly of microvascular origin and are functionally distinct from large vessel–derived endothelial cells. Therefore, to better address this issue, we performed expression tyrosine kinase profile using HDMECs to both verify and extend our previous observations related to ErbB2 and other tyrosine kinase expression. Monomers and dimeric, pentameric, and octameric procyanidin fractions isolated from cocoa were used in the human dermal microendothelial cell treatments for the kinase profiling experiments. Cells were grown in complete media with 10% FCS, VEGF, bFGF and EGF. Then, endothelial candidate kinases that were modulated were selected only if they were differentially expressed at different doses and if they demonstrated reproducibility.

In Figure 1, the *Rsa*I digestion product mapped to ErbB2 is almost completely down-regulated by the pentameric procyanidin and slightly down-regulated by the monomeric treatment, whereas the kinase Met is

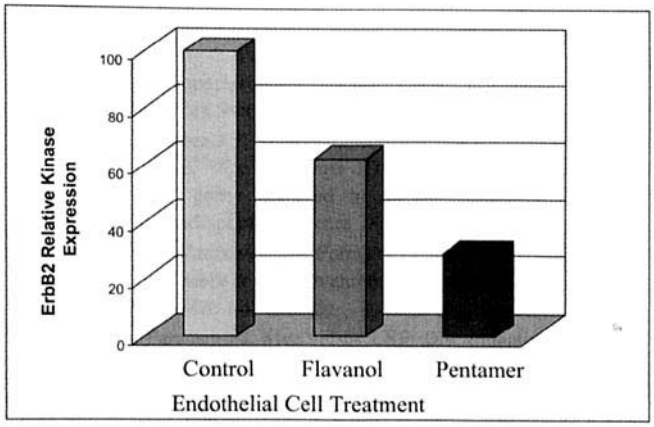

Figure 2. Quantitative real-time PCR experiments verifying reduced ErbB2 gene expression. Cells were stimulated with 1 μ*M* H_2O_2 for 30 mins and harvested after 4 hrs. A 20-μg/ml dose of both flavanol and pentameric fractions show decrease expression compared with that of the saline control. Beta-actin expression was used to normalize ErbB2 expression. The pentameric treatment is significantly reduced from the control treatment ($P < 0.001$). Results are reported from two quantitative real-time PCR experiments (n = 6) along with SEM bars.

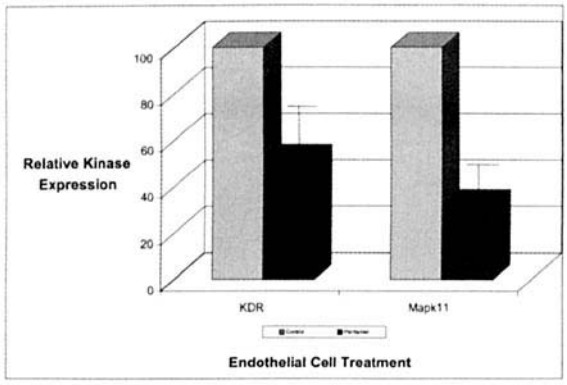

Figure 3. Quantitative real-time PCR experiments verifying reduced KDR and MAPK11 gene expression from 20 μg/ml pentamer procyanidin pretreated and control HDMECs. Cells were stimulated with 1 μ*M* H_2O_2 for 30 mins and harvested after 4 hrs. Beta-actin expression was used to normalize PCR expression results. Results are reported from two quantitative real-time PCR experiments (n = 6) along with SEM bars.

unaffected by either the cocoa flavanol (monomeric) or pentameric procyanidin treatments. ErbB2 also contains a *Bsp1286*I restriction site. Note that when the kinase PCR products were digested with *Bsp1286*I, a similar pattern of inhibition was exhibited (Fig. 1, right panel).

Quantitative real-time PCR was performed for verification using specific primers to ErbB2 at varying doses of oligomer. A dose response was performed and the optimal conditions were selected for all work. Results from these experiments show a significant decrease in expression by the 20 μg/ml dose of flavanol and pentameric procyanidin fractions (69 μ*M* for flavanol fractions and 14 μ*M* for pentameric fractions) compared with that of the saline control (Fig. 2). Because ErbB2 may involve expression of VEGF and MAPK system, further real-time PCR data revealed a decrease inVEGFR-2/KDR, the main mitogenic kinase receptor for VEGF and Mapk11/p38beta2 (Fig. 3).

Previous gene expression experiments demonstrated a maximal decrease in expression after >24 hrs of treatment. To determine whether this differential modulated gene expression of tyrosine kinase receptors would result in changing the signaling patterns of angiogenic-challenged HDMECs, we ran Western blots of HDMEC lysates using a panel of antityrosine phosphoantibodies. The relevancy of this observation is important in establishing a mechanism in retarding downstream growth signaling. For example, the angiogenesis process has many redundant systems linked to downstream signaling proteins that promote growth. By interfering with ErbB2, other components could continue the growth signaling.

Flavanols and procyanidins may also interfere with the phosphorylation of these downstream proteins. In Figure 4A and B, we obtained a protein tyrosine phosphorylation pattern of resting and angiogenic-stimulated pentameric procyanidin treated and control cells using two different pantyrosine phosphoantibodies (Py-20 and RC20H). Before angiogenic treatment, several proteins appeared to be slightly differentially phosphorylated between the control and treated cells. Twenty minutes posttreatment with 1.0 μ*M* H_2O_2, the control cells showed proteins phosphorylated around 129 kDa, whereas in the same area, the pentameric procyanidin–treated cells had little phosphorylation. We are

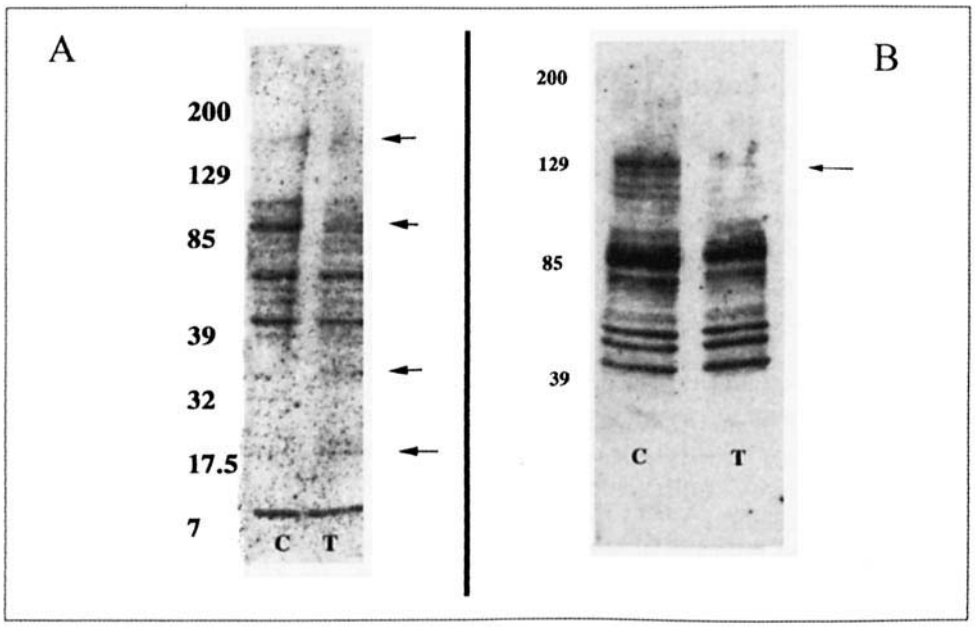

Figure 4. Western blot using protein lysates of 50 μg/ml pentamer procyanidin pretreated (48 hrs) and control HMDECs. (A) The blot was probed with recombinant HRP-anti-phospotyrosine (Py-20). (B) This blot was probed with recombinant HRP-anti-phospotyrosine (RC20H). Arrows indicate mild differences in tyrosine phosphorylation between control (C) and procyanidin-treated (T). Protein samples were standardized by having relatively equal staining to antitublin proteins. In (B), treated cells have reduced tyrosine phosphorylation around 129 kDa compared with that of controls.

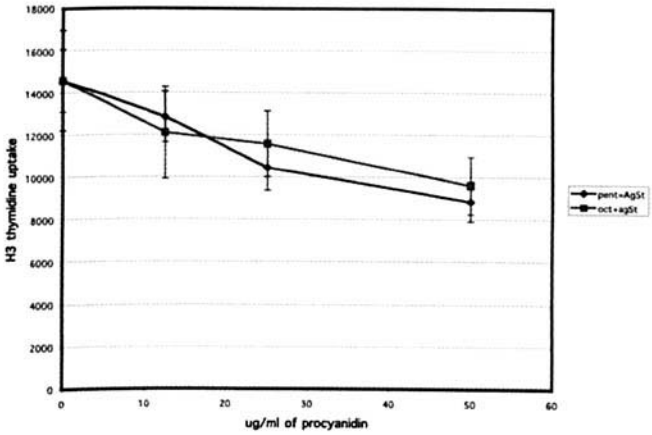

Figure 5. Proliferation of HMDECs treated with varying concentrations of pentamer and octamer procyanidin oligomers. Cells were exposed to 1 μM H_2O_2 for 30 mins and harvested after 48 hrs. Both pentamer and octomer show dose-dependent inhibition of growth. Results are CPM of tritium uptake from triplicate samples along with SEM bars.

performing additional experiments to identify the molecular targets of angiogenesis that were not demonstrated by tyrosine kinase gene expression profiling.

To assess the role of cocoa procyanidins in modulating proliferation of HDMECs, experiments were conducted to determine whether the pentameric or octameric (or both) cocoa procyanidin fractions also changed the growth characteristics of HDMECs under angiogenic stimulation. HDMECs were grown in serum-free defined media and treated with the cocoa procyanidin fractions. Cells were stimulated with 1.0 μM H_2O_2 and then assayed for proliferation after 48 hrs. Both the pentameric and octameric cocoa procyanidin fractions inhibited the proliferation of endothelial cells in a dose-dependant manner. The 50 μg/ml treatment for both procyanidin fractions achieved 30% inhibition of proliferation compared with nontreated control (Fig. 5). In a parallel experiment, 10 ng/ml of recombinant VEAGF (rVEGF) was added to the media after H_2O_2 stimulus. The oligomer-treated cells continued to manifest decreased proliferation. No significant changes were observed between the rVEGF cultures and VEGF-free cultures. The cells were monitored for viability, and no significant reduction in cell viability was observed.

Discussion

We report herein the effects of purified cocoa flavanols and procyanidins on tyrosine kinase expression. We also suggest possible, subsequent physiological implications and note that the molecular changes observed may be secondary to changes in cellular protein tyrosine phosphorylation of signaling proteins. This thesis is supported by data obtained from our analysis of tyrosine kinase gene expression, phosphorylation patterns, and endothelial cell proliferation. Although the concentrations of the procyanidins used herein are likely to exceed the physiological range obtainable *in vivo*, the potential implications of these compounds in the biochemistry and cycle of human cells warrant further studies.

Flavanols and procyanidins derived from cocoa have been shown to have antioxidant properties and to modulate gene expression; both mechanisms may in turn provide protection of the endothelium from oxidative stress and endogenous ROS stimulation of angiogenesis. In this respect, the endothelial cells involved in tumor angiogenesis, wound healing, and inflammation are predominantly of microvascular origin and are functionally distinct from large vessel–derived endothelial cells, which have been largely used for *in vitro* vascular research. We chose HDMECs as a model based on their sensitivity to proangiogenic stimuli such as VEGF or oxidative stress. Several factors have been suggested in the regulation of angiogenesis, including hypoxia, oxidative events, and the activation of oncogenes such as ras, raf, and src (27, 28). We note that cellular signaling through the ErbB receptors plays an important role in this scenario, with specific tyrosine kinases acting on such receptors ultimately regulating angiogenesis (29–31) and through an up-regulation of VEGF (31, 32). Purified cocoa procyanidins decrease the expression of tyrosine kinase receptor ErbB2, one of the important members of the ErbB family of kinases that includes EGFR (HER1/ErbB1), ErbB2 (HER2/Neu), ErbB3 (HER3), and ErbB4 (HER4; Ref. 33). The inhibition of the ErbB2 gene reduces the formation of functionally more active heterodimers, thus also decreasing downstream signaling events that lead to more angiogenic stimuli. We cannot rule out, however, that VEGF may be indirectly inhibited by pentameric procyanidin treatments; the alteration in ErbB2 signaling may in fact reduce endogenous VEGF production. Overexpression of ERbB2 or an activating mutation of ErbB2 (or both) can be found in patients with breast and ovarian cancer (34).

ErbB receptors are coupled to several kinase signaling pathways, including ERK1/2 (p44/p42) MAPKs, phospholipase C, phosphatidylinositol 3-kinase, and c-Jun NH2-terminal kinase (35). We demonstrate herein that HDMECs treated with purified cocoa flavanols and pentameric procyanidins have an altered tyrosine kinase phosphorylation profile compared with that of untreated cells, thus suggesting a concurrent suppression of angiogenic signaling. Three differentially phosphorylated proteins were found. The identification of these proteins and their respective pathways will be crucial in future studies and will confirm the mechanism proposed.

We also showed that intracellular ROS up-regulated ErbB2, whereas low amounts of H_2O_2 stimulate angiogenesis in the same cell model. The ability of cocoa flavanols and procyanidins to decrease lipid peroxidation and scavenge ROS could lead to the observed phenomena. Similarly, the decrease of ROS-mediated signaling could influence the expression of proangiogenic genes such as ErbB2. It is interesting that H_2O_2 also increased the ets-1 mRNA level, an important transcription factor that has also

been shown to be involved in angiogenesis. Alternatively, we propose that the down-regulation of ErbB2 gene transcription by cocoa flavanols and procyanidins may be independent from their antioxidative properties. Procyanidins have been linked to nonredox-type interactions such as enzyme inhibition that involves flavanoid–protein-binding interactions. Enzymes that have been implicated include ornithine decarboxylase, S-adenosylmethionine decarboxylase, and xanthine oxidase (36). Moreover, some procyanidins can affect enzymes involved in lipid metabolism (37). These examples of procyanidin-protein interaction open the possibility that these compounds may act as potential agonists, or antagonists, to proteins involved in angiogenesis signaling or gene expression.

In summary, we have demonstrated that purified pentameric and octameric cocoa procyanidin fractions inhibit the proliferation of HDMECs, as well as the activation of several proteins (via reduced tyrosine phosphorylation) in angiogenic signaling. We also confirmed that pentameric cocoa procyanidins down-regulate the expression of ErbB2 tyrosine kinase in HDMECs. Our results indicate that specific cocoa procyanidins can act as an antiangiogenic factor in this *in vitro* model. It is important to question whether this is a specific phenomenon of endothelial cells and whether this model might be applicable to other cell types, including neophilic types. If this is the case, the use of such dietary compounds may prove useful as an adjuvant in the prevention or treatment of tumor onset and progression.

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