Pentameric Procyanidins Isolated from *Theobroma cacao* Seeds Selectively Downregulate ErbB2 in Human Aortic Endothelial Cells

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Flavonoids isolated from cocoa have biological activities relevant to oxidant defenses, vascular health, tumor suppression, and immune function. The intake of certain dietary flavonoids, along with other dietary substances such as tocopherols, ascorbate, and carotenoids, is epidemiologically associated with a reduced risk of cardiovascular disease. Flavonoids have also been shown to modulate tumor pathology *in vitro* and in animal models. We took advantage of the conserved sequences found in tyrosine kinases to study the influence of cocoa fractions and controls on gene expression. We report that the pentameric procyanidin (molecular weight of 1442 daltons) fraction isolated from cocoa was a potent inhibitor of tyrosine kinase ErbB2 expression, a receptor important in angiogenesis regulation. Consistent with this primary observation, the cocoa flavonoid fraction also suppressed human aortic endothelial cell (HAEC) growth and decreased expression of two tyrosine kinases responsive to ErbB2 modulation, namely VEGFR-2/KDR and MapK 11/p38β2. These inhibitory effects were observed when HAECs were treated with the flavonol fraction (molecular weight 280 daltons) isolated from cocoa, which comprise the structural subunits from which the procyanidin flavonoid subclass is biosynthetically constructed. Downregulation of ErbB2 and inhibition of HAEC growth by cocoa procyanidins may have several downstream implications, including reduced vascular endothelial growth factor (VEGF) activity and angiogenic activity associated with tumor pathology. These results suggest specific dietary flavonoids are capable of selectively inhibiting ErbB2 and therefore may offer important insight into the design of therapeutic agents that target tumors overexpressing ErbB2. Exp Biol Med 229:255–263, 2004

Key words: oxidative defense; ErbB2; procyanidin; flavonoids; vascular endothelial growth factor

Theobroma cacao is a tree of equatorial origin that has a rich history of providing traditional medicines used widely in Central America and Western Europe (1). The majority of these medicinal preparations were derived from the seeds of its fruit, which are commonly referred to as cocoa beans. Depending on postharvest handling and processing, cocoa and its derivative products (e.g., chocolate) can contain extraordinary amounts of flavonols and procyanidins, which are structurally related subclasses of the broader class of natural products known as flavonoids (2). Figure 1 illustrates the chemical structures of flavonols and a procyanidin pentamer. The flavonols and the procyanidins isolated from cocoa have been shown to possess biological activities relevant to oxidant defenses, vascular health, tumor suppression, and immune function (3–10). The chronic ingestion of flavonol- and procyanidin-rich cocoa is associated with a reduction in low-density lipoprotein (LDL) oxidation and inhibition of platelet function *ex vivo* (11–17). In addition, specific procyanidin fractions isolated from cocoa have demonstrated protection against *in vitro* peroxynitrite-mediated protein damage as well as against oxidation of synthetic liposomes and DNA (18–21). *In vitro* studies with peripheral blood mononuclear cells have shown that purified cocoa procyanidin fractions,

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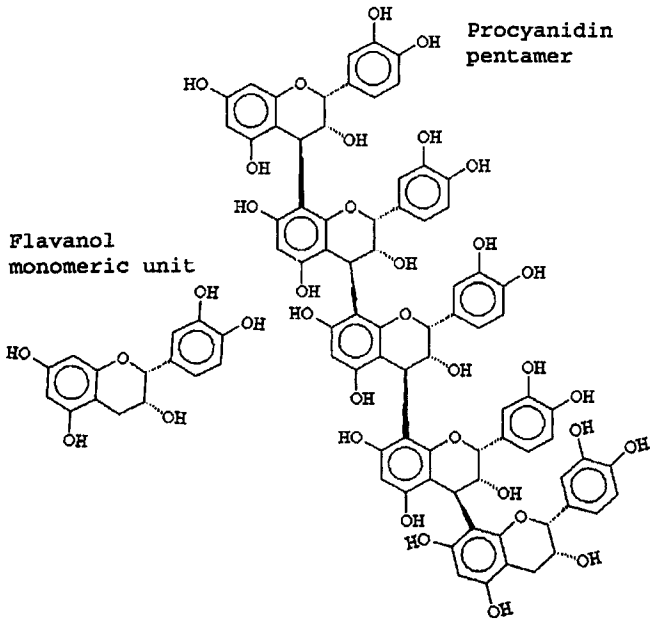

Figure 1. Chemical structures for epicatechin/catechin moiety and a representative procyanidin pentamer.

as well as a crude cocoa extract, can alter cytokine transcription (22–24), suggesting that these natural products have the potential to modulate the immune response. These mechanistic observations may provide a basis for the substantial body of epidemiological literature that suggests that the regular consumption of foods rich in flavonoids, such as those found in tea and certain cocoas and chocolates, is associated with a reduced risk of coronary heart disease, stroke, and certain cancers (25–29).

We used a unique profiling expression assay to determine the effects of purified cocoa flavonol and pentameric procyanidin fractions on tyrosine kinase expression. This method helped to identify several important kinases that were found to be differentially expressed. Pentameric procyanidin–treated human aortic endothelial cells (HAEC) also showed a decrease in proliferation. The results from this study present the possibility that specific cocoa flavonoids could influence important angiogenic and oncogenic tyrosine kinase genes at the transcriptional level, which may result in decrease proliferation of endothelial cells.

Materials and Methods

Cocoa Fraction Preparation. Cocoa flavonol and pentameric procyanidin fractions were provided by Mars, Inc. (Hackettstown, NJ), and were characterized, isolated, and purified according to the normal-phase chromatographic method of Adamson *et al.* (2).

Treatment of Cells. For the kinase profiling experiments, varying concentrations of the purified cocoa fractions and controls (2.5–40 μg/ml) were incubated with 75% confluent HAEC in Medium 200 media (Cascade Biologics, Inc., Portland, OR) with 10% fetal calf serum (FCS) supplemented with 2% (v/v) hydrocortisone, 1 ng/ml human epidermal growth factor (EGF), 10 ng/ml basic fibroblast growth factor (bFGF), and 3 ng/ml heparin for three different time periods (2, 8, 24 hrs). Positive and negative controls were run simultaneously. Cells were monitored at each harvest time point for changes in morphology, growth, and viability. Viability was measured by comparing the amount of adherent cells verses the amount of sloughed-off cells. Supernatant at the end of the time points was removed from the wells, and sloughed cells were counted. Trypsinized adherent cells were removed from the wells, an aliquot was counted, and the two values were then compared.

RNA Isolation. Cells were harvested for each time point and immediately lysed by the addition of 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA). RNA was isolated and frozen at −80°C. Five micrograms of RNA was used for reverse transcription using Superscript II RT (Invitrogen).

Kinase Profiling. The kinase profiling assay was previously established by Robinson *et al.* (30) and Kung *et al.* (31) and was carried out in the present study with slight modifications. Essentially, various tyrosine kinase transcripts were amplified with degenerate primers derived from conserved motifs (DFG and DVW) within the catalytic domains. The 5′ (sense) primer used to encode the amino acid sequence K [V/I][S/C/G] DFG was represented by 5′-AAR RTT DCN GAY TTY GG. The 3′ (antisense) primer

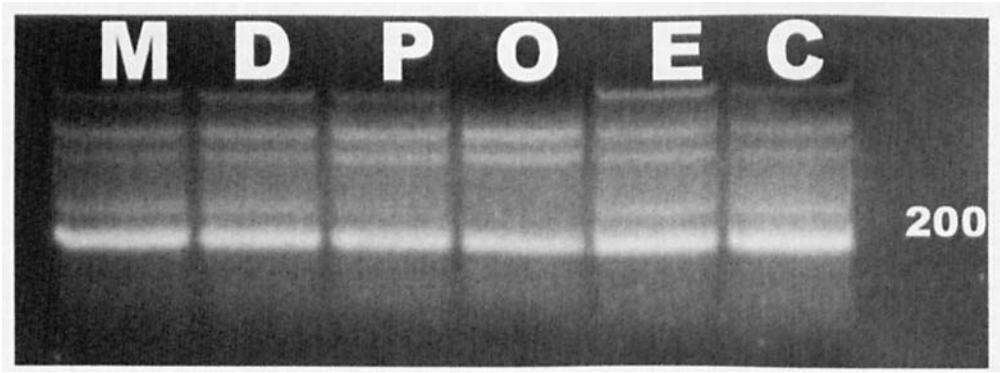

Figure 2. Kinase profile PCR of cDNA from cocoa treatments of human aortic endothelial cells. Note the kinase band at 170 bp. Lanes: M, cocoa flavonol fraction; D, dimeric cocoa procyanidin fraction; P, pentameric cocoa procyanidin fraction; O, octomeric cocoa procyanidin fraction; E, (–)-epicatechin standard; and C, control saline. This illustrates that there is relatively equal kinase transcript expression for each sample.

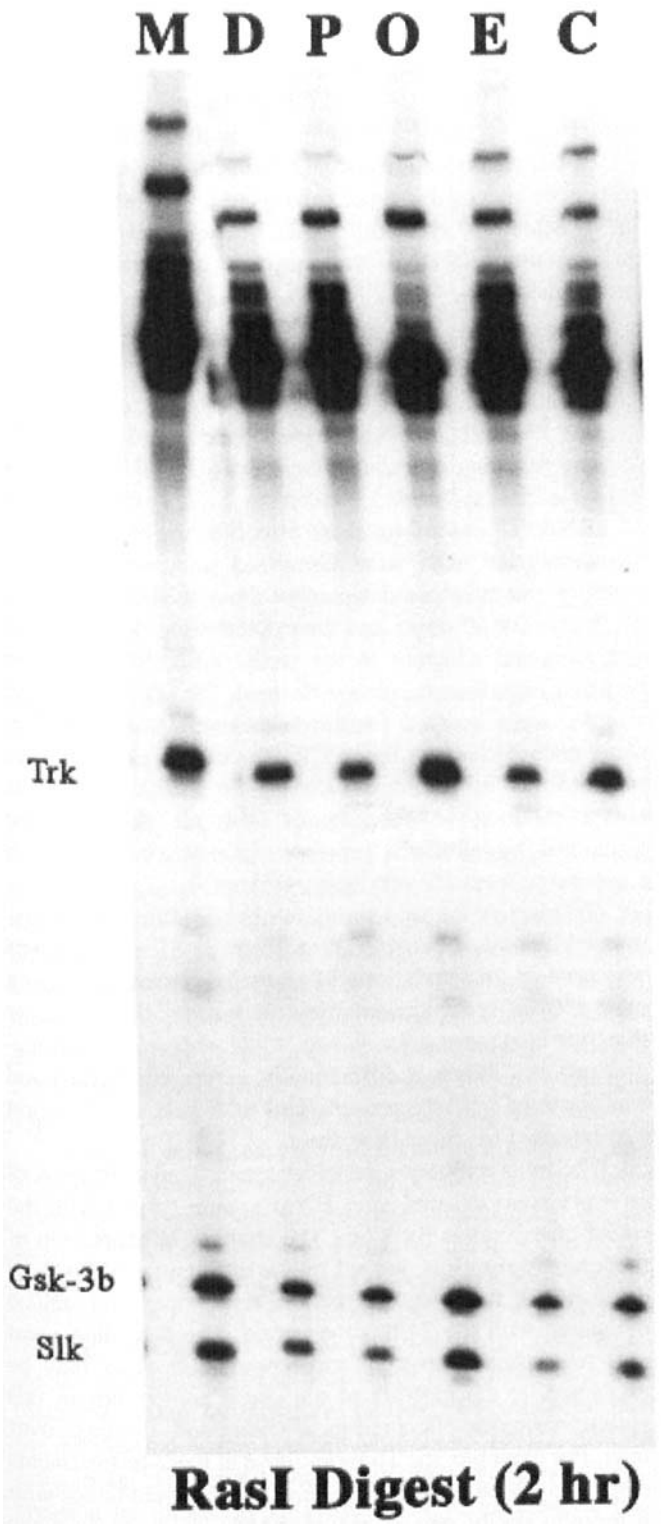

Figure 3. Autoradiograph showing example of tyrosine kinase PCR *Rsa*I digest products from human aortic endothelial cell cocoa flavonol– and procyanidin-treated cultures. Digest bands representing tyrosine kinase TRK, GSK-3b, and SLK are identified. There appears to be only minor fluctuations in expression in response to treatments. Lanes: M, cocoa flavonol fraction; D, dimeric cocoa procyanidin fraction; P, pentameric cocoa procyanidin fraction; O, octomeric cocoa procyanidin fraction; E, (–)-epicatechin; and C, control saline.

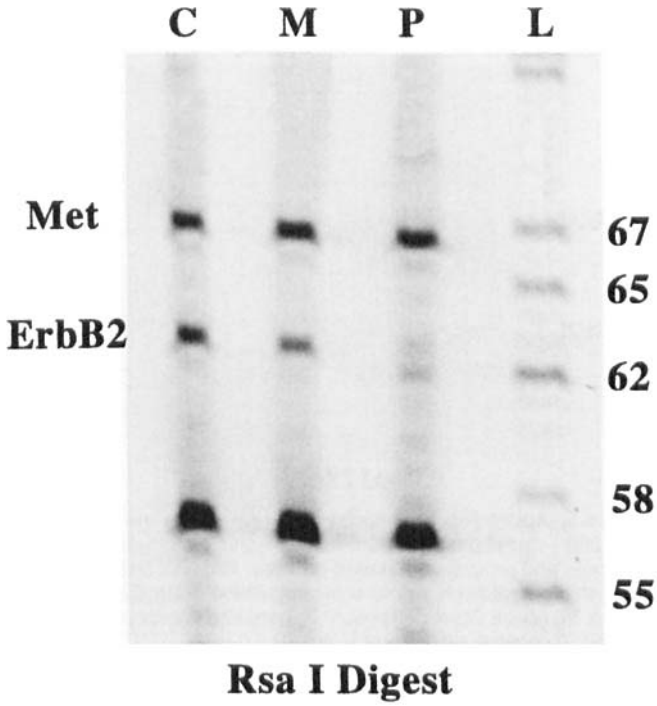

Figure 4. Autoradiograph showing example of tyrosine kinase PCR *Rsa*I digest products from human aortic endothelial cell cocoa flavonol- and procyanidin-treated cultures. Digest bands representing tyrosine kinase MET and ErbB2 are identified. Cocoa flavonol and pentameric procyanidin treatments show down regulation of ErbB2 expression. Lanes: M, cocoa flavonol fraction; P, pentameric cocoa procyanidin fraction; C, control saline; and L, ladder.

used to encode the amino acid sequence DVW [S/A][F/Y] was represented by 5′-RHA IGM CCA IAC RTC. The mixed bases were defined as follows: N = A + C + T + G, D = A + T + G, H = A + T + C, R = A + G, Y = C + T, M = A + C, and I = deoxyinosine. The 5′ primer was labeled with [γ-^{33}P]-ATP (NEN Life Science Products, Boston, MA) catalyzed by a T4 polynucleotide kinase (Invitrogen). The PCRs were conducted with [^{33}P]labeled 5′ primer and unlabeled 3′ primer using AmpliTaq Gold DNA polymerase (Perkin Elmer, Boston, MA). The PCR annealing temperatures were initially set at 45°C for five cycles followed by 55°C for the final 25 cycles. The PCR-amplified products from HAEC samples were electrophoresed in a 2.4% agarose gel (3:1 ratio of Nusieve GTG to agarose LE; BMA, Rockland, ME). The 153–177 bp bands were excised from the gel and DNA purified using the QIAEX II gel extraction kit (QIAGEN, Valencia, CA). The activity of the eluted DNA was determined by liquid scintillation counting, and each sample was normalized to 20,000 cpm/μl by dilution with nuclease-free H_2O. 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/bis-acrylamide; Bio-Rad Laboratories, Hercules, CA). The gel was then dried and subjected to autoradiography.

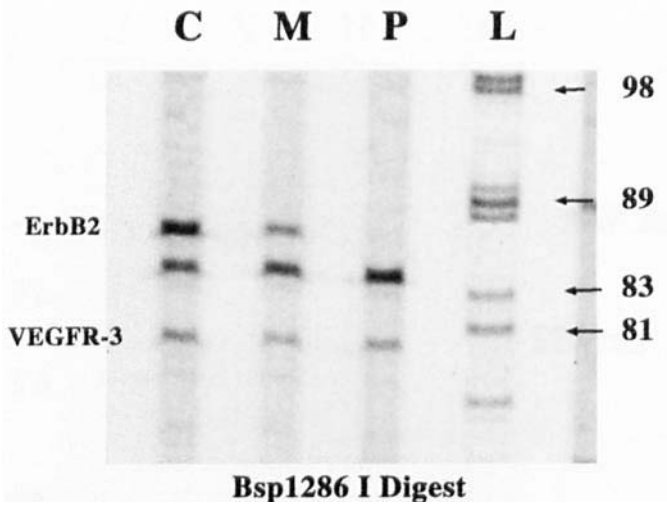

Figure 5. Autoradiograph showing example of tyrosine kinase PCR Bsp1286 I digest products from human aortic endothelial cell cocoa flavonol– and procyanidin-treated cultures. Flavonol and pentameric procyanidin treatments reflect down regulation of ErbB2 expression. Lanes: M, cocoa flavonol fraction; P, pentameric cocoa procyanidin fraction; C, control saline; and L, ladder.

A detailed computerized restriction digest map for over 100 tyrosine kinase (TC) was prepared for all of the known commercially available restriction enzymes. By examining the image of the gel, differentially expressed kinase bands can be mapped for each digest. Using the sequence ladder that is run with each gel, a base pair size was assigned to each differentially expressed band. The size of the band produced by a given enzyme was then compared with predicted fragments from the known kinases based on virtual restriction enzyme mapping.

Quantitative Real-Time PCR. Quantitative real-time PCR was performed using the GenAmp 5700 Sequence Detection System (PE Biosystems, Foster City, CA). Briefly, optimal PCR primers were designed for each kinase using Primer Express software (PE Biosystems) to specifically amplify the gene of interest. Primer sequences for ErbB2 were forward 5′-AGGGAAAACACATCCCCCAA, reverse 5′ TTGGCAATCTGCATACACCAG; KDR forward, 5′-CTTCCAAGTGGCTAAGGGCA, reverse 5′-GGCGAGCATCTCCTTTTCTG; MapK 11 forward, 5′-ACGCCCGGACATATATCC, reverse 5′-GTCCAGCACCAGCATCCT. Primers for housekeeping genes (Actin or GAPDH) were purchased from Perkin Elmer. PCR was performed using the SYBR Green technology. Triplicate 50-µl PCR reactions for each sample and control contained 5 µl of optimally diluted cDNA sample, 3 µl each of optimal forward and reverse primers, 5 µl of 10X SYBR PCR buffer, 4 µl of dNTP blend (2.5 mM), 6 µl of 25 mM $MgCl_2$, 0.25 µl of AmpliTaq Gold, and 23.75 µl of H_2O. Standard curves were prepared for both target and housekeeping genes from pre-prepared reference samples. The relative concentration of the target gene was determined from the standard curve and normalized to the amount of housekeeping gene for each sample.

Proliferation. To assess cell proliferation, HAEC (2.5×10^4 cells/15 mm wells) were cultured with Human Endothelial–Serum Free Medium 200 (Cascade Biologics) supplemented with EGF and FGF (10 ng/ml) in the absence or presence of VEGF (10 ng/ml) as indicated. Endothelial cells were treated with cocoa oligomers and controls for 24 and 48 hrs. At this time, cells were pulsed with 0.6 µCi [^{3}H]thymidine/15 mm well for 12 hrs, and [^{3}H] thymidine incorporation into DNA was measured as an index of cell proliferation (32).

Results

If cells are compromised by the toxicity of reagents, tyrosine kinase PCR-based expression experiments can be skewed, resulting in variable numbers of viable cells. This can give rise to yields of differing orders of magnitude of mRNA. To control for these effects in the current study, the endothelial cells were visualized microscopically for viability and possible detachment from wells. Cells from treatments for all doses and time points were >90% viable and remained adherent to the wells. Also, before kinase profiling experiments were performed, the cDNA from the samples were assayed for the housekeeping gene β-actin using quantitative real-time PCR. The cDNA samples were standardized using these values. The [^{33}P]labeled PCR kinase products (170 bp band) from all samples were visualized by ethidium bromide staining, and they had comparable intensities and cpm values (Fig. 2).

To identify the maximum number of differentially expressed kinases, a panel of 30 different restriction enzymes was used in this study, providing the means of identifying over 150 different kinases. Several kinases possess more than one enzyme restriction site, which aids in the verification process. When a differentially expressed digest band was observed from the sequencing PAGE gels, the digestion was repeated for reproducibility.

The most numerous, consistent, and intense differences in expression occurred after HAECs were treated with the cocoa procyanidins for 8 hrs. The changes in expression of the kinase transcripts, judged by the differential intensity of the bands at the 8-hr time period, were more pronounced compared with the 24-hr time period. The 8-hr time point gels were more consistent and reproducible. This may be explained by the half-life of the cocoa compounds in this system where the effects of these compounds diminish over time. For example, the 10 µg/ml dose at the 2-hr treatments displayed a total of eight differentially expressed bands with a reproducibility rate of six of eight (75%), whereas the 8-hr treatment displayed 20 differentially expressed bands with a reproducibility rate of 18 of 20 (90%). Overall, 90% of these differentially expressed bands occurred when comparing the control treatment with the pentameric cocoa procyanidin treatment. From these results, endothelial candidate kinases modulated by treatment were selected for further analysis if they were differentially expressed at different doses and time points, and if they demonstrated

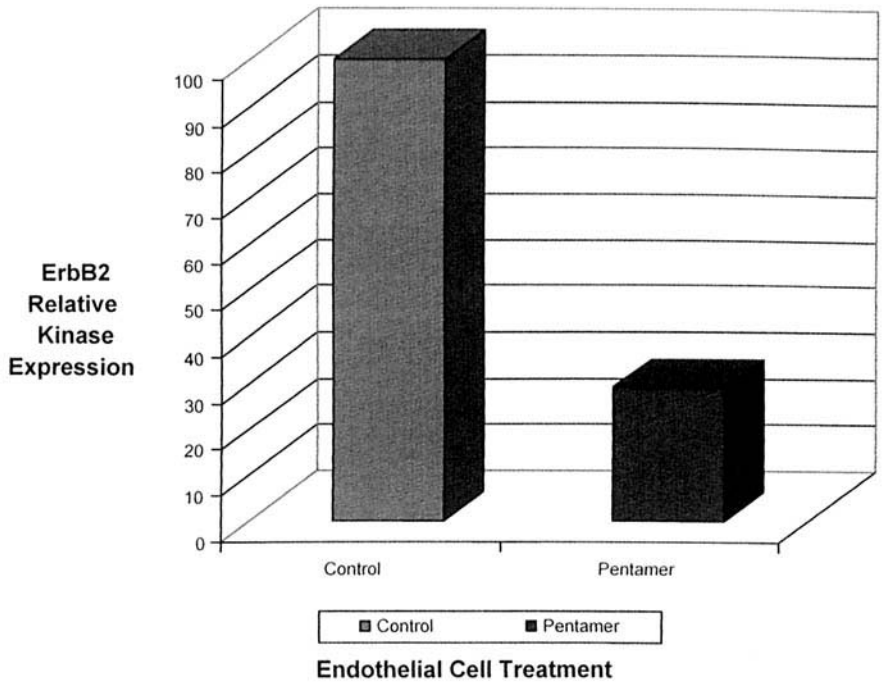

Figure 6. Quantitative real-time PCR experiments verifying reduced ErbB2 expression. Beta-actin expression was used to normalize ErbB2 and control PCR expression results.

reproducibility. A few select kinases—Trk, Gsk-3b, and Slk—appear to be unaffected by treatment with cocoa procyanidins after *Rsa*I digestion (Fig. 3). These unaffected bands were used as internal controls to authenticate the efficiency of the digest and the relative level of expression between the different kinases. It should be noted that with the use of degenerate primers in kinase profiling PCR, each individual kinase sequence may amplify with differing efficiency. In other words, two different kinases with equal expression can display bands with dissimilar intensities.

Figure 4 shows that the *Rsa*I digestion product mapped to ErbB2 decreased intensity by the pentameric procyanidin treatment, whereas the kinase Met is unaffected by the flavonol treatment. Because ErbB2 contains a Bsp1286 I restriction site when the kinase PCR products were digested with Bsp1286 I, a similar pattern of inhibition was exhibited (Fig. 5). Similar results were obtained when aortic endothelial cells isolated from a different individual were used (data not shown). Quantitative real-time PCR was performed for verification using specific primers to ErbB2. Results from these experiments show a significant decrease in expression by the 20 μg/ml dose (8 hrs) of pentameric procyanidins compared with the saline control (Fig. 6). Since ErbB2 may involve expression of VEGF and the MAPK system, further real-time PCR experiments revealed a decrease in VEGFR-2/KDR, the main mitogenic kinase receptor for VEGF and MapK 11/p38β2 (Fig. 7).

Proliferation experiments were conducted to determine whether the procyanidins also changed growth characteristics of HAECs. Human aortic endothelial cells were grown in serum-free-defined media without VEGF, but with EGF treated with the pentameric cocoa procyanidins, and then assayed for proliferation. The pentameric procyanidin treatment inhibited the proliferation of endothelial cells compared with the control and flavonol treatments. When 10 ng/ml of VEGF was added to the pentameric procyanidin-treated cultures, the cells could not be rescued by the addition of VEGF and continued to show decreased proliferation (Fig. 8).

Discussion

Tyrosine kinases are intimately involved in regulating a variety of biological processes related to vascular endothelium. Therefore, we thought it important to evaluate their expression in response to dietary agents known to demonstrate the strong potential for conferring vascular health benefits, namely cocoa flavonols and procyanidins (5, 7–9, 11, 13, 15, 28). Flavonoids, including select procyanidins present in cocoa, have been studied as chemopreventive agents in regard to tumorigenesis, tumor growth, and angiogenesis (4, 33–36). Many cellular factors contribute to support tumor growth. In general, increased angiogenesis is a hallmark of tumor formation, and disruption of the angiogenic process has been shown to have therapeutic value. Metabolic and epidemiological studies indicate that the regular intake of certain polyphenols, like those found in cocoa, increases the plasma level of antioxidants and may reduce the deleterious effects of certain reactive oxygen (ROS) and nitrogen (RNS) species (37–39). The thrust of this paper is both *in vitro* and also to demonstrate the power of our

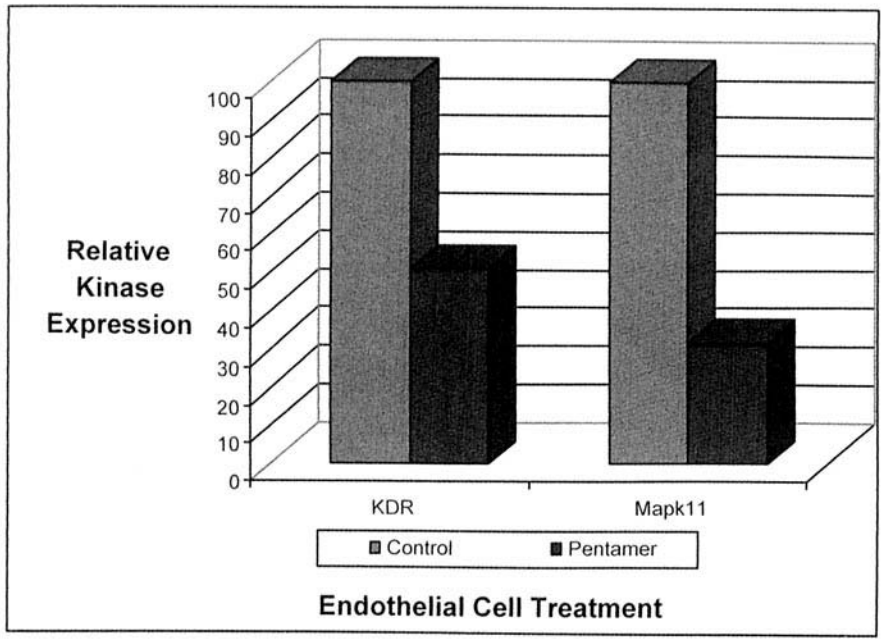

Figure 7. Quantitative real-time PCR experiments verifying reduced KDR and MapK 11 expression. Beta-actin expression was used to normalize KDR and MapK 11 and control PCR expression results.

high throughput analysis using kinase profiling (RAGE). Specific discussion on the bioavailability of procyanidins is discussed elsewhere (37–39). Reactive oxygen species have been linked to both angiogenesis and cancer (40). Recent studies have indicated other mechanisms may be involved in chemoprevention besides the antioxidative effects of polyphenols (35, 36, 41). The present study explored the possibility that specific cocoa flavonoids could influence important angiogenic and oncogenic tyrosine kinase genes at the transcriptional level.

The selective inhibition of ErbB2 expression by pentameric cocoa procyanidins found in the kinase profiling, as well as the quantitative real-time PCR experiments, offers a further understanding of the mechanisms underlying the biological actions of certain dietary flavonoids. ErbB2 is overexpressed in several human cancers, including colon, breast, lung, ovarian, and prostate. Patients who overexpress ErbB2 have a higher rate of relapse and shorter survival. ErbB2 signaling is associated with several kinase pathways (MAPK and PI-3/Akt) and with the polyamine synthesis pathway. Studies suggest that increased polyamine biosynthetic activity critically interacts with ErbB2 in promoting human mammary cell transformation in culture and that the MAPK cascade is an important mediator of this interaction (42). Previously, it has been shown that procyanidin-enriched cocoa extracts caused a 70% growth inhibition of Caco-2 cells, a colon cancer cell line, with a blockade of the cell cycle at the G2/M phase. This study also found significant decreases of ornithine decarboxylase and S-adenosylmethionine decarboxylase activity, two key enzymes of polyamine biosynthesis (33). It is reasonable to speculate that in the Carnesecchi *et al.* (33) experiment, ErbB2 expression was affected by the cocoa procyanidins and subsequently disrupted the activity of the two polyamine synthesis enzymes. Our results are consistent with those of Pianetti *et al.* (35), who showed downstream inhibition of signaling of PI-3/ATK by 80 μg/ml of the green tea flavonol, epigallocatechin-3 gallate, in breast cancer cells.

In the current study, we show a significant decrease in ErbB2 expression accompanied by inhibition of cell growth HAECs treated with pentameric cocoa procyanidins. This inhibition of growth was seen using doses of 20 μg/ml. Expression experiments were conducted with cells grown in complete growth media with serum. The cocoa procyanidin–treated cells, overriding proexpression growth factor feedback systems, observed a 75% decrease of ErbB2 transcripts. The promoter region of the c-erb B2/neu gene contains sequences that dictates regulatory responses to several environmental signals, and it is highly inducible. Transcriptional regulators, including PEA3 and the adenovirus Type 5 E1A, are able to repress ErbB2 gene expression (43). It is thought that transcriptional repressors that downregulate ErbB2 can be effective regimens for cancer treatment. Both ErbB2 and VEGF can activate the MAPK kinase system. Consistent with the reduced expression of these factors, we observed a down regulation of MapK 11 in the current study.

Angiogenic processes can occur by activating both vascular endothelial growth factor (VEGF) receptors and epidermal growth factor (Erb) receptor signaling. The interaction between the ErbB tyrosine kinase receptors and their ligands plays an important role in angiogenesis via the regulation of autocrine and paracrine loops. It has been reported that overexpression of the ErbB2 receptor results in

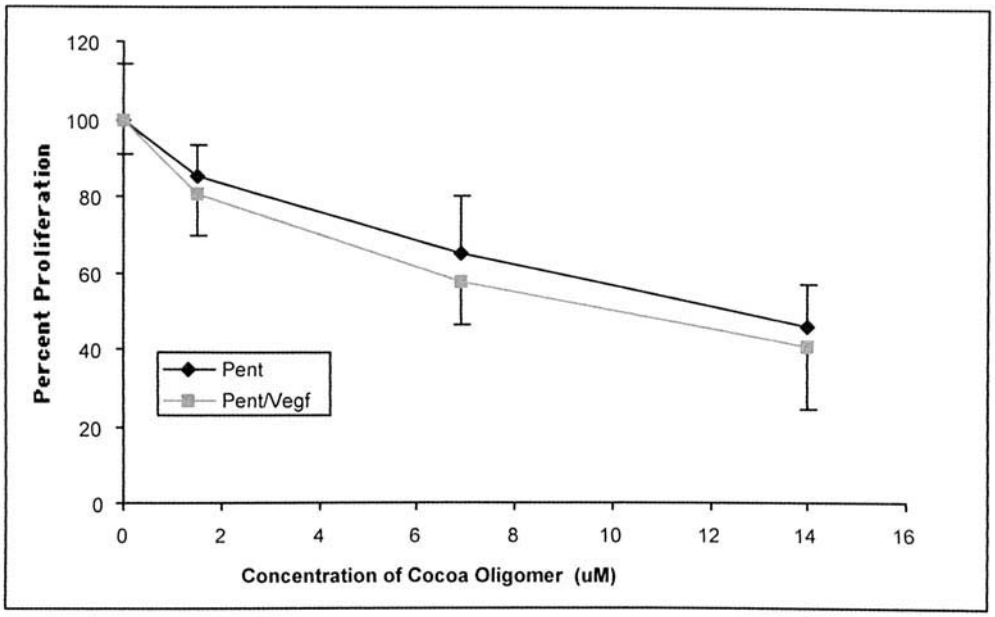

Figure 8. Proliferation of human aortic endothelial cell cultures treated with 14.0, 7.0, and 1.4 μM of cocoa procyanidins in serum-free-defined media in the presence and absence of VEGF (10 ng/ml). Treatment with the pentameric cocoa procyanidins produce a dose-dependent inhibition of growth as measured by ^{3}H-thymidine uptake. Added VEGF failed to rescue cells.

induction of the basal level of VEGF, and exposure to heregulin—the ligand for ErbB3 and ErbB4—further enhances VEGF secretion (44). Thus, lowering levels of ErbB2 may reduce growth by one mechanism while inhibiting the efficiency of VEGF production by not being available for dimerization with activated ErbB3 and ErbB4. Since both ErbB2 and VEGF can activate the MAPK kinase system, it is important to see the downstream effects of the procyanidins influence on ErbB2. We also observed down regulation of MapK 11 and KDR (VEGF receptor), which is consistent with the down regulation of ErbB2 and leads to growth inhibition. We note that our data is on gene expression only. Further, there is no suitable ErbB2 antibody available for Western blotting. However, work in progress will describe protein expression.

These results suggest that certain cocoa procyanidins can selectively act at the transcriptional level, and that they may have downstream effects on factors influencing both endothelial and tumor angiogenesis. It is reasonable to speculate that the observed effects of pentameric cocoa procyanidins on ErbB2, VEGFR-2/KDR, and MapK 11/p38β2 may contribute to the putative anticarcinogenic properties often attributed to certain dietary flavonoids. It is important to note here that while cocoa flavonols are well absorbed (17, 45), the procyanidins are not. The absorption of dimer has been reported to occur in both humans (17, 28, 45, 46) and experimental animals (45, 47), but larger oligomers have not yet been reported. However, the longer oligomers can reach high micromolar concentrations throughout the gut (48); thus the results presented in the current paper may be particularly important with respect to gastrointestinal cancers. If the effects of longer oligomers in the gut can result in systemic effects is currently unknown. Finally, it is critical to note the observation that the cocoa flavonol fraction had minimal effects on ErbB2, VEGFR-2/KDR, and MapK 11/p38β2, in contrast to the pentameric cocoa procyanidin fraction, and underscores the concept that dietary flavonoids are capable of modulating important biological pathways in a manner that is dependent on their specific structures. This observation suggests that many of these compounds may have useful pharmaceutical applications. In particular, we suggest that the pentameric procyanidins in cocoa are a novel class of candidate molecules capable of therapeutically modulating ErbB2.

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