JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Modulation of Inflammatory Gene Expression by a Bilberry (*Vaccinium myrtillus* L.) Extract and Single Anthocyanins Considering Their Limited Stability under Cell Culture Conditions

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ABSTRACT: Studies with nonintestinal models indicate that anthocyanin-rich extracts can modulate inflammatory gene expression and may help prevent development of inflammatory bowel diseases (IBD). This work investigated the influence of a bilberry (*Vaccinium myrtillus* L.) extract (BE) and comprising anthocyanins on pro-inflammatory genes in IFN-γ/IL-1β/TNF-α stimulated human colon epithelial cells (T84) by qRT-PCR and cytokine arrays. Moreover, the stability of selected anthocyanins under cell culture conditions was examined to assess their anti-inflammatory properties. BE and single anthocyanins significantly inhibited the expression and secretion of IBD-associated pro-inflammatory mediators (TNF-α, IP-10, I-TAC, sICAM-1, GRO-α) in the stimulated cells. The anti-inflammatory activity thereby strongly depends on the aglycon structure (hydroxylation and methylation pattern) and the sugar moiety. In contrast to anthocyanidins, which were highly unstable in cell culture medium, suggesting that their degradation products might contribute to the inhibitory effects assigned to the parent compounds, anthocyanins have higher stability and may directly contribute to BE's effects.

KEYWORDS: *bilberry (Vaccinium myrtillus L.) extract, anthocyanins, inflammatory gene expression, inflammatory bowel disease (IBD), cytokines, stability*

INTRODUCTION

Anthocyanins are naturally occurring, water-soluble secondary metabolites that are responsible for the blue, purple, and red colors of many plant tissues. The most common natural anthocyanidins are cyanidin (cy), delphinidin (del), petunidin (pet), peonidin (peo), pelargonidin (pel), and malvidin (mal), bound to glucose (glc), galactose (gal), rhamnose (rha), xylose (xyl), or arabinose (ara) as 3-*O*-glycosides or 3,5-*O*-diglycosides.[1] The chemical structures of primary anthocyanidins and their common abbreviations are shown in Figure 1 and Table 1.

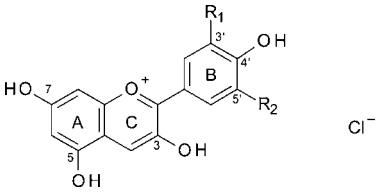

Figure 1. Chemical structures of anthocyanidins as derivatives of the 2-phenylbenzopyrylium (R1, R2 = H, OH, or OCH_3).

Table 1. Nomenclature of Anthocyanidins and Their Common Abbreviations As Shown in Figure 1

anthocyanidin	R_1	R_2
pelargonidin (pel)	H	H
cyanidin (cy)	OH	H
delphinidin (del)	OH	OH
peonidin (peo)	OCH_3	H
petunidin (pet)	OCH_3	OH
malvidin (mal)	OCH_3	OCH_3

Anthocyanins are among the heavily consumed classes of flavonoids, the main dietary sources being berries (e.g., bilberries or raspberries), vegetables, and red wine.[2,3] Estimated human daily intakes range from 12 mg/day in the United States[4] to 126 mg/day in Scandinavian countries,[5] and one of the richest dietary sources is bilberry (*Vaccinium myrtillus* L.).[6] The chemical structure, stability, and color of anthocyanidins strongly depend on the pH. They are stable at pH $\leq$3, at which they occur as flavylium cations, but at higher pH (and hence cell culture conditions) anthocyanins and their corresponding anthocyanidins have limited stability,[7,8] resulting in the formation of hydroxycoumarin glycosides and ring-opened chalcones or the respective phloroglucinol aldehydes and corresponding phenolic acids. Furthermore, anthocyanins readily degrade or react with other constituents in the media to form colorless or insoluble brown complexes. The presence of an oxonium ion adjacent to carbon at position 2 makes anthocyanins particularly susceptible to nucleophilic attack by various compounds and degradation by both various enzymes (e.g., polyphenol oxidase, PPO) and high temperatures.[9]

Despite their limited stability, anthocyanins have attracted interest in recent years due to their diverse biological activities, including antioxidant and anticarcinogenic activities,[10,11] and apparent preventative effects on various degenerative diseases.[12] Several lines of evidence suggest that they can modulate

Special Issue: 5th International Conference on Polyphenols and Health

Received: March 23, 2012
Revised: August 11, 2012
Accepted: August 22, 2012
Published: August 22, 2012

dx.doi.org/10.1021/jf3028842 | *J. Agric. Food Chem.* 2012, 60, 8902−8910

inflammatory gene expression in vitro and in vivo, and both anthocyanin-rich extracts and single major anthocyanins have anti-inflammatory effects in nonintestinal models.[13−16] Thus, they may also inhibit processes in the intestinal epithelium linked to the development of inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC). IBD are generally characterized by excessive release of pro-inflammatory cytokines and chemokines (e.g., TNF-α, IP-10, and IL-8) from cells of the intestinal immune system, notably intestinal epithelial cells (IEC).[17] IEC form the first immunological barrier, on the villus surface, to pathogens and potentially damaging compounds in the intestine. Following exposure to luminal antigens they initiate mucosal infiltration with leukocytes by releasing cytokines and chemokines.[18] In this context, the intestinal epithelium plays a pivotal role in IBD pathogenesis by regulating local inflammation and immune responses, through interactions with components of the intestinal immune system. Indeed, analyses of bacterial−enterocyte interactions have revealed that IL-8 is one of several host factors released by IEC along with IL-1β, IL-6, TNF-α, GM-CSF, and several C-X-C and C-C chemokines following bacterial challenge.[19−21] This mixture of cytokines and chemokines ensures that circulating immune cells are not only chemoattracted to mucosal sites of inflammation but also activated and sustained to produce further cytokines which stimulate and maintain the inflammatory process in IBD. Thus, this cell population might be a promising target for dietary anti-inflammatory intervention providing that these substances are available at intestinal sites of inflammation. However, to optimize their use further knowledge of their activities, structure−activity relationships, action mechanisms, and stability is required.

Hence, the objectives of the present study were to elucidate the effects of an anthocyanin-rich bilberry extract (BE), and component anthocyanins, on the expression of IBD-associated pro-inflammatory marker genes (TNF-α, IP-10, IL-8) in human colon epithelial cells (T84) by quantitative real-time PCR. We also examined the ability of the compounds to inhibit production and secretion of multiple inflammatory cytokines and chemokines using a human cytokine array and the stability of selected anthocyanins under cell culture conditions to assess whether degradation products might contribute to effects assigned to the parent compounds.

MATERIALS AND METHODS

Cell Line, Media, Chemicals, and Stimuli. Human colon T84 (ATCC CCL-248) cells, derived from a lung metastasis of colon carcinoma, were obtained from the American Type Culture Collection (Rockville, MD, USA). DMEM (high glucose) and Ham's Nutrient Mixture F12 (Ham's F12) cell media and the supplements glutamine, penicillin/streptomycin, and fetal calf serum (FCS) were from Invitrogen (Karlsruhe, Germany), and catalase (bovine liver) was from Sigma-Aldrich (Taufkirchen, Germany). All solvents and chemicals used were of analytical grade or met cell culture standards. Bilberry (*V. myrtillus* L.) extract was obtained from Kaden Biochemicals (Hamburg, Germany). Del, mal, peo, pet, cy, and pel (chlorides) were purchased from Extrasynthése (Lyon, France). 3-*O*-Glucosides, galactosides, and arabinosides of del, mal, peo, pet, and cy (designated x-3-glc, x-3-gal, and x-3-ara, respectively, where x is the acglycone) and del-3,5-*O*-diglucoside (del-3,5-glc) were obtained from Extrasynthése, Polyphenols Laboratories AS (Sandnes, Norway), and Cfm e.K. (Marktredwitz, Germany). All anthocyanin and anthocyanidin standards used had at least 96% purity (up to 99%). Acetonitrile (MeCN, HPLC grade) and hydrochloric acid (37%) were purchased from J. T. Baker (Phillipsburg, NJ, USA), formic acid (puriss. p.a. ≥ 98%) and dimethyl sulfoxide (DMSO) from Merck (Darmstadt, Germany), and 3-hydroxyphenylacetic acid (3-HPA, ≥ 99%), 2,4,6-trihydroxybenzaldehyde (≥97%), 3,4-dihydroxybenzoic acid (≥97%), and saponin from Sigma-Aldrich. Human recombinant cytokines TNF-α (Biochrom AG, Berlin, Germany), IL-1β (Axxora, Lörrach, Germany), and IFN-γ (BioVision, Milpitas, CA, USA) were stabilized after resuspension by adding 0.1% bovine serum albumin (in Dulbecco's phosphate-buffered saline, DPBS). Cell culture equipment (e.g., cell culture flasks, Petri dishes, well plates, etc.) was purchased from Greiner Bio-One (Essen, Germany).

Cell Culture and Stimulation Protocols. T84 cells were maintained in 75 cm^2 flasks in DMEM/Ham's F12 (1:1, v/v) medium supplemented with 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine in a 5% CO_2 humidified incubator at 37 °C. Initially cells were seeded in six-well plates in 2 mL of fresh medium per well at 5×10^5 cells/mL. Cells were starved for 16 h in DMEM/Ham's F12 (1:1, v/v) with 0.5% FCS after 48 h of cultivation and then pretreated with test compounds dissolved in DMSO in serum-free medium containing 100 units/mL catalase for 1 h. They were subsequently stimulated with a cytokine mixture (CM) consisting of 10 ng/mL TNF-α, 5 ng/mL IL-1β, and 10 ng/mL IFN-γ for 4 or 16 h (the compounds remain in the medium together with the cytokines) prior to qRT-PCR and proteome profiling, respectively. The final DMSO concentration in all mixtures, including blanks, was 0.5%.

Cell Viability Testing. The cytotoxic effects of BE and the individual anthocyanins/anthocyanidins listed above on T84 cells were determined using a resazurin reduction assay,[22] as follows. Cells were seeded in 48-well plates at 1.5×10^5 cells/well in 0.5 mL of the medium described under Cell Culture and Stimulation Protocols. After 24 h of cultivation, the single substances (at 25−300 μM concentrations) or BE (10−500 μg/mL) were added, and then after a further 4 and 24 h of incubation, respectively, at 37 °C and 5% CO_2, the test medium was removed, cells were washed with PBS, and 0.5 mL of resazurin solution (44 μM, 37 °C) was added per well. After a final 1 h of incubation at 37 °C and 5% CO_2, the concentration of remaining resazurin was analyzed fluorometrically, using excitation and emission wavelengths of 544 and 590 nm, respectively. The cytotoxicity of the test substances was expressed as the percentage reduction of resazurin in cell suspensions with test substances relative to the reduction in solvent controls (0.5% DMSO). The reduction of resazurin in medium controls, positive controls (0.1% saponin), and cell-free controls was measured in parallel.

Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis. Following stimulation, cells were lysed and total cellular RNA was prepared using Total RNA Isolation Reagent (ABgene, Hamburg, Germany) following the manufacturer's protocol. RNA samples were dissolved in DEPC-treated water and stabilized by adding RNA-Later reagent (Invitrogen, Karlsruhe, Germany). Dissolved RNA was immediately stored in a −80 °C freezer until use. Expression levels of inflammatory genes in the test cells were then quantified by reverse transcription PCR using aliquots of total RNA, as follows. After determination of the quantity and quality of isolated RNA using an ND-1000 spectrophotometer (NanoDrop Technologies, USA), cDNA was prepared from 1 μg of total cellular RNA per sample by reverse transcription using an iScript cDNA synthesis kit from Bio-Rad (Munich, Germany) according to the manufacturer's instructions. PCR products were then synthesized from the cDNA (100 ng) using ABsolute qPCR SYBR Green (ABgene, Hamburg, Germany), and transcript levels of investigated genes were determined by qRT-PCR using an iCycler (Roche, Mannheim, Germany), following the manufacturer's recommendations, and gene-specific primers (from MWG-Biotech AG, Ebersberg, Germany) for human IP-10 (forward, 5′-TGAGCCTACAGCAGAGGAA-3′; reverse, 5′-TACTCCTTGAATGCCACTTAGA-3′), IL-8 (forward, 5′-TGCCAAGGAGTGCTAAAG-3′; reverse, 5′-CTCCACAACCCTCTGCAC-3′), TNF-α (forward, 5′-TCTTCTGCCTGCTGCACTTTGG-3′; reverse, 5′-ATCTCTCAGCTCCACGCCATTG-3′), and GAPDH (forward, 5′-CCTCCGGGAAACTGTGG-3′; reverse, 5′-AGTGGGGACACGGAAG-3′). The amplification protocol consisted of initial denaturation

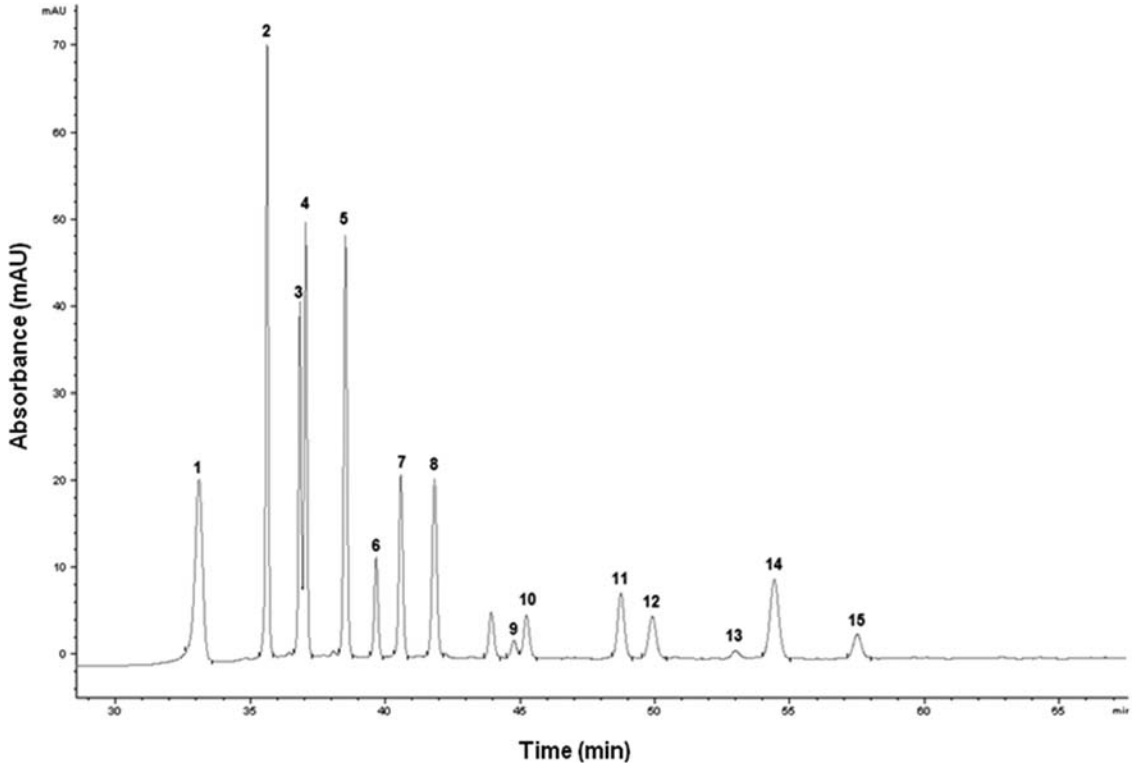

Figure 2. HPLC-DAD chromatogram obtained by monitoring the bilberry (*Vaccinium myrtillus* L.) extract (25 μL injection of a 100 μg/mL solution) at 520 nm: del-3-gal (1), del-3-glc (2), cy-3-gal (3), del-3-ara (4), cy-3-glc (5), pet-3-gal (6), cy-3-ara (7), pet-3-glc (8), peo-3-gal (9), pet-3-ara (10), peo-3-glc (11), mal-3-gal (12), peo-3-ara (13), mal-3-glc (14), mal-3-ara (15).

at 95 °C for 10 min followed by 45 cycles of 15 s denaturation at 94 °C, 30 s annealing at 56 °C, and 30 s extension at 72 °C. Relative mRNA amounts were calculated from the real-time PCR efficiencies and crossing point deviations of unknown samples (target genes) versus an internal control, here the housekeeping gene *GAPDH*, using the model proposed by Pfaffl.[23]

"Proteome Profiler" Human Cytokine Antibody Array Analysis. To assess correlations between reductions in the levels of the investigated genes' transcripts and the encoded proteins induced by the tested compounds, selected cytokines and chemokines were analyzed using the "Human Cytokine Array Panel A" antibody array (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany; see Table 3), as follows. After 48 h of cultivation, T84 cells were starved for 24 h in DMEM/Ham's F12 (1:1) medium with 0.5% FCS, incubated for 1 h with test compounds dissolved in DMSO in serum-free medium containing 100 units/mL catalase, and induced for 16 h with a human cytokine mixture (CM) consisting of 10 ng/mL TNF-α, 5 ng/mL IL-1β, and 10 ng/mL IFN-γ. After a short centrifugation, the supernatant was then used, following the array manufacturer's protocol (http://www.rndsystems.com/pdf/ARY005.pdf). The assay has been verified in a further independent experiment (full experiment carried out on a different day).

Stability of Selected Anthocyanins and HPLC-DAD Analysis of BE. To assess the influence of cells on the stability of the selected anthocyanins, T84 cells (maintained as described under Cell Culture and Stimulation Protocols) were seeded in 48-well plates in 0.5 mL of fresh medium per well (at 300,000 cells/mL). After 24 h of cultivation, the culture medium was replaced by serum-free DMEM/Ham's F12 1:1 (v/v) medium, supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 100 units/mL catalase, 100 μM of the test substances, and 0.5% DMSO. After predefined incubation periods (0−24 h) in a 5% CO_2 humidified incubator at 37 °C, aliquots were taken from the incubation mixtures (and blanks with 0.5% DMSO), acidified with hydrochloric acid (final pH, 1.8), and immediately frozen in liquid nitrogen to prevent further degradation. Samples were then stored in a −80 °C freezer until analysis. For cell-free controls the same protocol was used without cultured cells. All incubations were performed in triplicate, in at least three independent experiments.

After the samples had thawed, a solution containing two internal standards (0.08 g/L del-3,5-glc chloride and 0.2 g/L 3-hydroxyphenylacetic acid) dissolved in H_2O/MeCN/HCOOH (87:3:10, v/v/v) was added (1:10, v/v). The samples were then analyzed by high-performance liquid chromatography (HPLC) with diode array detection using an Agilent series 1200 system equipped with a photodiode array detector (DAD), a cooled autosampler (Agilent, Waldbronn, Germany), and a Luna 3u C18 (2) (250 × 4.6 mm, 3 μm) reversed-phase column (Phenomenex, Torrance, CA, USA). Anthocyanins, degradation products, and internal standards were then separated and eluted using a mixture of solvents A (H_2O/MeCN/HCOOH; 87:3:10, v/v/v) and B (H_2O/MeCN/HCOOH; 40:50:10, v/v/v), at a flow rate of 0.5 mL/min: the proportion of B was raised (linearly at each step) from 2 to 14% (0−10 min), held at 14% until 15 min, raised from 14 to 15% (15−20 min) and from 15 to 18% (20−30 min), held at 18% B until 35 min, raised from 18 to 99% B (35−37 min), and then held at 99% B until 57 min to wash the column before returning to 2% B (57−59 min) and re-equilibrating with 2% B (59−64 min). In each case, the injection volume was 25 μL; spectra were recorded from 200 to 600 nm; analytes in the eluent were monitored at wavelengths of 260, 274, 290, 360, and 520 nm; the autosampler was set at 4 °C; and the thermostatic column compartment was set at 40 °C.

Levels of anthocyanins and degradation products, and their limits of quantification (LOQ) and determination (LOD), were calculated using external calibration curves with standard solutions. Analytes were identified according to their retention times and the corresponding UV−visible spectra. Lyophilized BE was dissolved in mobile phase A and diluted to a final concentration of 100 μg/mL prior to HPLC analysis, as described above.

Statistical Analysis. Statistical evaluations of the data acquired in the cytotoxicity and stability assays were performed using Student's paired *t* tests or independent *t* tests, and differences between

compounds (and controls) were considered to be significant at $p \leq 0.05$ or $p \leq 0.01$, respectively. Gene expression data were deemed to indicate significant induction or repression when differences in mRNA levels between compared samples were ≥2-fold (log2). Data are presented as means of triplicate incubations from at least three independent experiments (full experiment carried out on a different day) ± standard deviation (SD).

RESULTS

HPLC-DAD Analysis of BE. Fifteen anthocyanins were detected in the BE by HPLC-DAD analysis, the most abundant being del-3-gal, del-3-glc, del-3-ara, cy-3-gal, and cy-3-glc (Figure 2), at concentrations (mg/g extract) presented in Table 2, which also shows the concentrations (μM) of the substances in 25 μg/mL BE extract (used in incubations with cells).

Table 2. Anthocyanin Concentrations in the BE and Corresponding Initial Concentrations in in Vitro Incubations with (for Illustration) 25 μg/mL Extract

anthocyanin	concentration in BE[a] (mg/g)	incubation concentration[b] (μM)
del-3-glc	46.8	2.5
cy-3-glc	46.2	2.6
del-3-ara	37.1	2.1
cy-3-gal	27.8	1.5
del-3-gal	27.2	1.5
pet-3-glc	18.6	1.0
cy-3-ara	18.2	1.1
mal-3-glc	14.5	0.7
pet-3-gal	9.7	0.5
pet-3-ara	8.6	0.5
peo-3-glc	8.0	0.4
mal-3-ara	5.9	0.3
mal-3-gal	5.0	0.3
peo-3-gal	1.6	0.1
peo-3-ara	0.9	0.1
total amount	276.1	15.1

[a]According to the manufacturer's specifications. [b]For an in vitro incubation with 25 μg/mL extract.

Cytotoxicity. At concentrations exceeding 100 μg/mL (BE) and 100 μM (single compounds) the test substances significantly reduced cell viability. After 4 and 24 h, 250 μg/mL BE reduced viability to 80 ± 5 and 60 ± 3% and 200 μM cy reduced it to 83 ± 2 and 78 ± 3%, whereas 200 μM del reduced it to 79 ± 1 and 63 ± 2%, respectively, relative to solvent controls. Cell viability in incubations with 200 μM of all other anthocyanidins (pel, peo, and mal) was >90% compared to solvent controls, and all investigated anthocyanins had only slight cytotoxic effects at 200 μM (cell viability > 95%) after 4 and 24 h of incubation. Generally, the anthocyanidins were more cytotoxic than the corresponding anthocyanins, and their cytotoxicity strongly depended on their hydroxylation and methoxylation patterns, declining in the order del > cy > pel > peo > mal (thus increasing with increases in hydroxylation). The maximum observed noncytotoxic concentrations have not been exceeded in further experiments using T84 cells.

Inflammatory Gene Expression. Expression ratios of the investigated genes (log2 ratios of transcript levels in induced versus uninduced cells (induction controls) and in induced, substance-treated versus induced, untreated cells, normalized to those of the internal reference, constitutively expressed, housekeeping gene GAPDH) are shown in Figures 3–6. In all experiments measured GAPDH mRNA levels did not vary significantly following treatment of the cells with either the CM or the applied test substances.

Table 3. Proteins Included in the Antibody Array and Their Coordinates in the Human Cytokine Antibody Array (Antibodies Spotted in Duplicates)

coordinate	detected protein	coordinate	detected protein
A1, A2	positive control	C7, C8	IL-13
A3, A4	complement component 5a	C9, C10	IL-16
A5, A6	CD154	C11, C12	IL-17
A7, A8	G-CSF	C13, C14	IL-17E
A9, A10	GM-CSF	C15, C16	IL-23
A11, A12	CXCL1	C17, C18	IL-27
A13, A14	CCL1	D3, D4	IL-32α
A15, A16	sICAM-1	D5, D6	CXCL10
A17, A18	IFN-γ	D7, D8	CXCL11
A19, A20	positive control	D9, D10	CCL2
B3, B4	IL-1α	D11, D12	MIF
B5, B6	IL-1β	D13, D14	CCL3
B7, B8	IL-1ra	D15, D16	CCL4
B9, B10	IL-2	D17, D18	PAI-1
B11, B12	IL-4	E1, E2	positive control
B13, B14	IL-5	E3, E4	CCL5
B15, B16	IL-6	E5, E6	CXCL12
B17, B18	IL-8	E7, E8	TNF-α
C3, C4	IL-10	E9, E10	STREM-1
C5, C6	IL-12 p70	E11, E12	negative control

Initial time course analysis of the selected pro-inflammatory marker genes' expression in T84 cells showed that they were all significantly up-regulated during the 24 h following stimulation

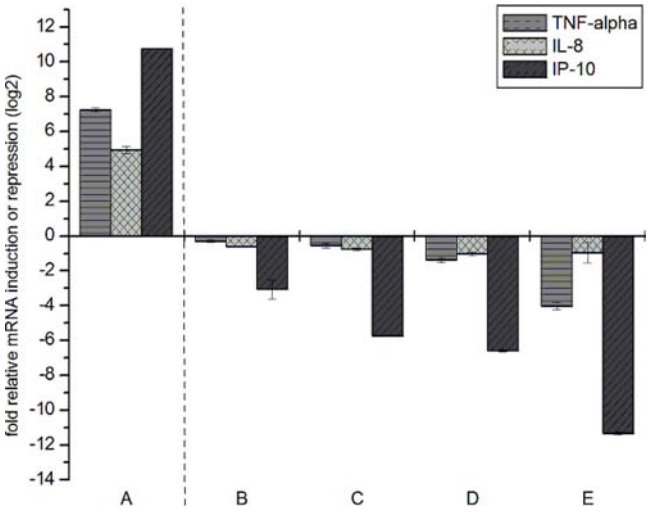

Figure 3. Influence of bilberry (*Vaccinium myrtillus* L.) extract on inflammatory gene expression in IFN-γ/IL-1β/TNF-α stimulated (4 h) human colon epithelial (T84) cells: fold changes (shown as log2 ratios) in transcript levels, normalized using transcript levels of the GAPDH gene, of indicated genes in uninduced cells relative to CM induction controls (A) and CM-stimulated cells pretreated with 2.5, 5, 10, and 25 μg/mL of the extract relative to CM-stimulated cells (B–E, respectively). Data shown are mean values ± SD obtained from at least three independent experiments.

dx.doi.org/10.1021/jf3028842 | J. Agric. Food Chem. 2012, 60, 8902–8910

with the CM (Figures 3–6A), and their mRNA levels were maximal 4 h after induction, on average. Thus, we used a 4 h induction time for the following co-incubations with test compounds.

In accordance with previous findings,[24] epigallocatechin-3-*O*-gallate (EGCG, a major flavonoid of green tea, used as a positive control) strongly inhibited the expressions of TNF-α and IP-10 in our cell model and moderately influenced IL-8 mRNA expression (data not shown).

BE also significantly and dose-dependently inhibited expression of the pro-inflammatory marker genes TNF-α and IP-10 in CM stimulated T84 cells, at concentrations of 2.5 and 25 μg/mL, respectively (Figure 3). It also slightly reduced transcript levels of the pro-inflammatory gene IL-8, but neither dose-dependently nor significantly at concentrations up to 25 μg/mL.

The effects of selected anthocyanins on the expressions of TNF-α, IL-8, and IP-10 genes in the stimulated T84 cells depended on both their aglycone and sugar moieties (Figures 4 and 5). IP-10 expression was significantly inhibited by cy-3-ara, the most potent inhibitor, cy-3-glc at 25 μM (the lowest concentration tested), and cy-3-gal at 50 μM. Peo-3-*O*-glycosides were active only as glucose conjugates in the tested concentrations (25–100 μM, Figure 6). Mal-3-glc, mal-3-gal, and pet-3-glc had no significant effect on expression of the investigated genes (data not shown). In contrast, mal-3-glc and mal-3-gal had slight inductive effects on IL-8 and IP-10 mRNA expression, respectively.

For further structure–activity analysis we also investigated the effects of the corresponding anthocyanidins (del, pet, cy, peo, and mal) on inflammatory gene expression in the stimulated T84 cells. As shown in Figure 6, cy, del, and pet significantly down-regulated IP-10 mRNA expression, but peo or mal did not (even at 100 μM, data not shown). Transcript levels of TNF-α were down-regulated by del and pet, but not by cy, peo, or mal (Figure 6). Cy significantly reduced TNF-α transcript levels at $\geq$50 μM, but peo or mal did not (at any tested concentration, data not shown). In contrast, the mRNA

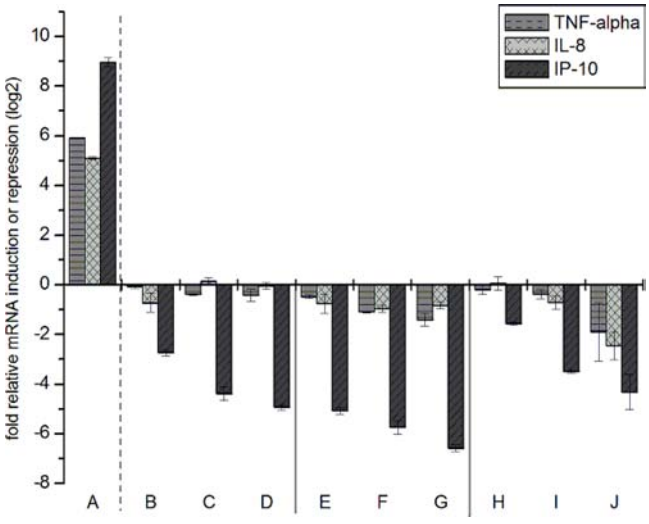

Figure 4. Influence of cyanidin-3-*O*-glycosides on inflammatory gene expression in IFN-γ/IL-1β/TNF-α stimulated (4 h) human colon epithelial (T84) cells: fold changes (shown as log2 ratios) in transcript levels, normalized using transcript levels of the GAPDH gene, of indicated genes in uninduced cells relative to CM induction controls (A) and CM-stimulated cells pretreated with the anthocyanins cy-3-glc at 25, 50, and 100 μM (B–D, respectively), cy-3-ara at these concentrations (E–G, respectively), and cy-3-gal at these concentrations (H–J, respectively), relative to CM-stimulated cells. Data shown are mean values ± SD obtained from at least three independent experiments.

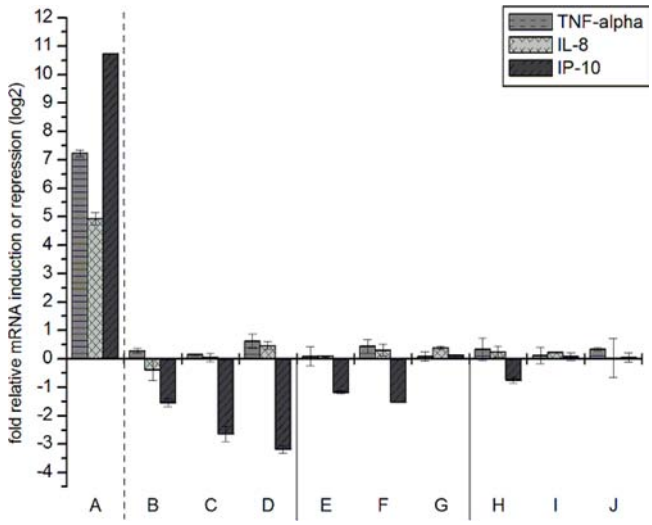

Figure 5. Influence of peonidin-3-*O*-glycosides on inflammatory gene expression in IFN-γ/IL-1β/TNF-α stimulated (4 h) human colon epithelial cells (T84): fold changes (shown as log2 ratios) in transcript levels, normalized using transcript levels of the GAPDH gene, of indicated genes in uninduced cells relative to CM induction controls (A) and CM-stimulated cells pretreated with the anthocyanins peo-3-glc at 25, 50, and 100 μM (B–D, respectively), peo-3-ara at these concentrations (E–G, respectively), and peo-3-gal at these concentrations (H–J, respectively), relative to CM-stimulated cells. Data shown are mean values ± SD obtained from at least three independent experiments.

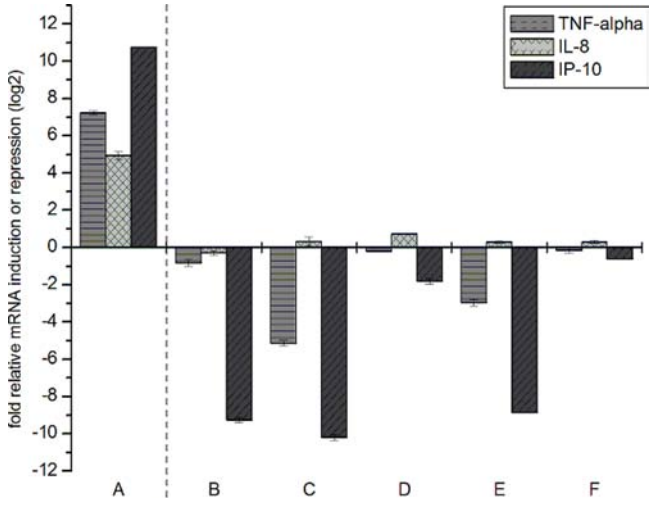

Figure 6. Influence of anthocyanidins on inflammatory gene expression in IFN-γ/IL-1β/TNF-α stimulated (4 h) human colon epithelial (T84) cells: fold changes (shown as log2 ratios) in transcript levels, normalized using transcript levels of the GAPDH gene, of indicated genes in uninduced cells relative to CM induction controls (A) and CM-stimulated cells pretreated with the anthocyanidins cy, del, peo, pet, and mal, each at 25 μM (B–F, respectively), relative to CM-stimulated cells.

dx.doi.org/10.1021/jf3028842 | J. Agric. Food Chem. 2012, 60, 8902–8910

expression of IL-8 was not significantly influenced by any investigated anthocyanidin.

Relative Levels of Pro-inflammatory Cytokines and Chemokines (Proteome Profiling). As shown in Figure 7II,

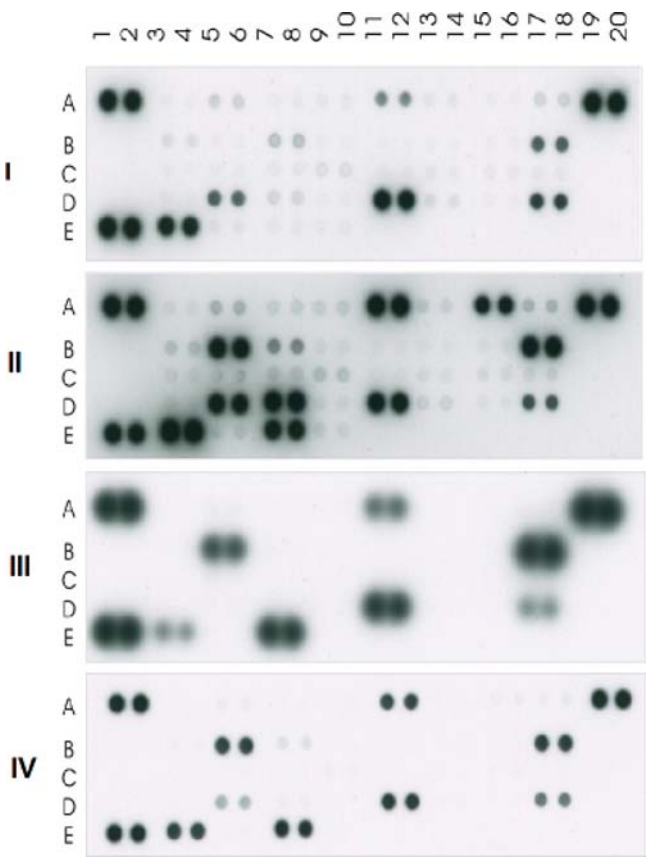

Figure 7. Influence of cyanidin and BE on translation and secretion of multiple released cytokines and chemokines in CM-stimulated (16 h) human colon epithelial (T84) cells investigated using a human cytokine antibody array (human cytokine array panel A, R&D Systems). Signals are from uninduced cells (I), induced cells (II), induced cells treated with 25 μM cyanidin (III), and induced cells treated with 25 μg/mL BE (IV).

stimulation of T84 cells with CM induced the synthesis of several pro-inflammatory cytokines, including not only IP-10 (CXCL10) and IL-8 but also RANTES (CCL5), I-TAC (CXCL11), GRO-α (CXCL1), and sICAM-1. Pretreatment with cy (25 μM) and BE (25 μg/mL) completely inhibited synthesis of IP-10, I-TAC, and sICAM-1 and slightly repressed GRO-α synthesis (Figure 7III,IV). The BE (but not cy alone) also slightly inhibited secretion of IL-8. Neither test substances influenced induction of the chemokine RANTES.

Stability of Selected Anthocyanins and Anthocyanidins under Cell Culture Conditions. The anti-inflammatory activities of the tested compounds depended on their sugar moieties (Figure 4) and the aglycone structure (Figure 6). This could have been due to either differences in their stability under cell culture conditions or steric effects of the conjugated sugar moieties. To test these possibilities we monitored the degradation of cyanidin-3-*O*-glycosides (cy-3-glc, cy-3-gal, and cy-3-ara) and the corresponding aglycone (cy) in T84 cell culture medium, with and without cells, by HPLC-DAD, as described under Stability of Selected Anthocyanins and HPLC-DAD Analysis of BE. Time courses of their degradation are shown in Figures 8 and 9 as percentages of the initial concentration (100 μM) and absolute amounts of the remaining substances, respectively.

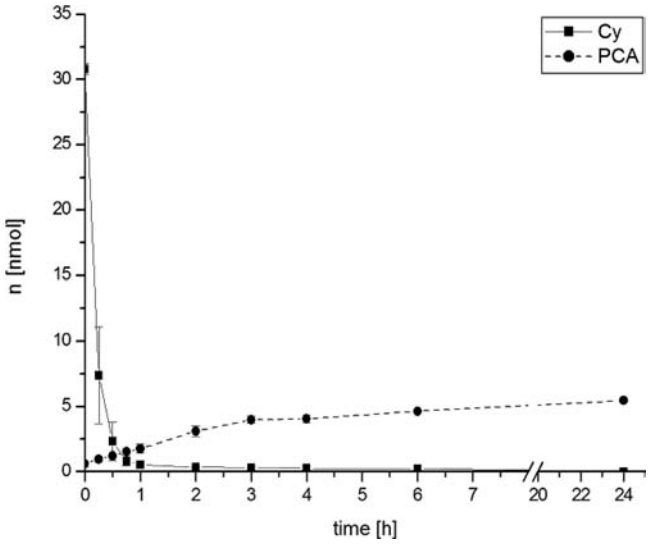

Figure 8. Twenty-four hour time course of the degradation of cyanidin (Cy) and formation of protocatechuic acid (PCA) in cell culture medium with T84 cells, shown as absolute amount of substance (n).

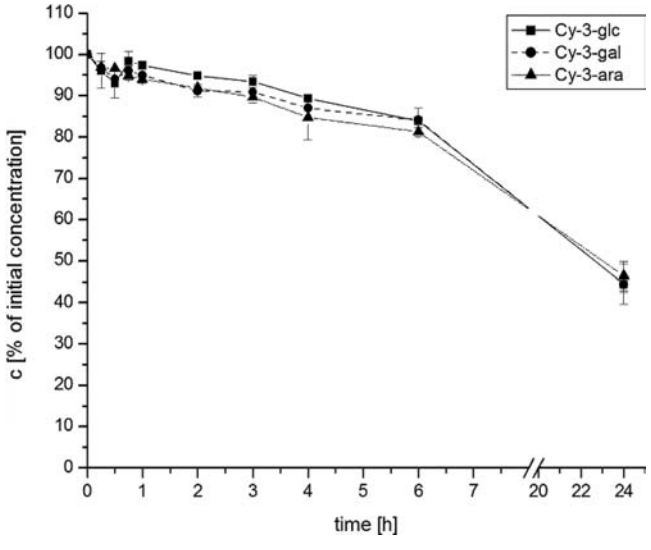

Figure 9. Twenty-four hour time course of the degradation of cyanidin-3-*O*-glucoside (cy-3-glc), cyanidin-3-*O*-galactoside (cy-3-gal), and cyanidin-3-*O*-arabinoside (cy-3-ara) in cell culture medium with T84 cells, shown as percentages of initial concentrations.

As shown in Figure 8, cy rapidly degraded in the cell culture medium, its concentration declining by ca. 75, 90, and 98.5% within 15, 30, and 60 min, respectively. The corresponding phenolic acid, protocatechuic acid, formed asynchronously; amounts equivalent to 5% of the initial cy concentration were detected after 60 min and ca. 20% (the highest level) after 24 h. Only traces, between the LOD (162 nM) and LOQ (270 nM), of the corresponding phloroglucinol aldehyde were detected. There was no significant difference in the cy degradation time courses between incubations with and without cells. In addition, we detected unknown metabolites and/or degradation products by HPLC-DAD analysis at 520 nm (data not shown).

dx.doi.org/10.1021/jf3028842 | *J. Agric. Food Chem.* 2012, 60, 8902−8910

The anthocyanins proved to be generally much more stable under our cell culture conditions than the corresponding aglycone. As shown in Figure 9, slight degradation of the investigated cyanidin-3-*O*-glycosides was detected; >90% of the parent compounds remained after 3 h and approximately 45% after 24 h incubations with cells. The expected degradation products, protocatechuic acid and phloroglucinol aldehyde, were only detected after 6 and 24 h. Furthermore, we detected unknown metabolites and/or degradation products by HPLC-DAD analysis at 260, 290, and 520 nm, different from those detected in incubations with cy. Interestingly, there were no significant sugar moiety related differences in the degradation of the investigated cyanidin-3-*O*-glycosides, in incubations either with no cells (data not shown) or with T84 cells (Figure 9). However, the cyanidin-3-*O*-glycosides degraded much more rapidly in cell-free incubations; in contrast to the incubations with cells, they were only stable for up to 1 h and were nearly completely degraded after 24 h (data not shown).

DISCUSSION

Anthocyanins have apparent ability to inhibit expression of pro-inflammatory genes in the intestine and thus are promising candidates for dietary interventions to prevent the development of IBD such as Crohn's disease and ulcerative colitis, provided they are sufficiently stable to reach intestinal sites of inflammation (see the Introduction).

A rich source of these substances is bilberry. Hence, we evaluated effects of a bilberry extract on IBD-associated pro-inflammatory gene expression in human colon epithelial cells (T84), induced by the cytokines IFN-γ, IL-1β, and TNF-α according to the method of Potoka et al.[25] This mixture of cytokines ensures an inflammatory response in IEC along with the induction of several pro-inflammatory genes according to IBD pathogenesis. For evaluating preventive effects on epithelial gene expression, we investigated the pro-inflammatory marker genes TNF-α, IP-10, and IL-8. The roles of these cytokines in the development of IBD linked to the intestinal epithelium have been extensively reviewed.[26]

BE and single anthocyanins significantly inhibited expression and secretion of the IBD-associated pro-inflammatory mediators TNF-α and IP-10. Moreover, the human cytokine array analysis showed that the expression and secretion of further pro-inflammatory cytokines, including I-TAC and GRO-α were inhibited in the stimulated cells. I-TAC and GRO-α also play a supplementary role in IDB pathogenesis.[27−29] The whole extract, but not cy alone, had a slight inhibitory effect on the secretion of IL-8. Hence, the effects on mRNA expression of IP-10 and IL-8 appear to be reflected in levels of the corresponding proteins (although the results should be confirmed by quantitative protein analysis, e.g., using ELISA, because such cytokine array analysis is semiquantitative). In addition to down-regulating pro-inflammatory chemokines and cytokines, the tested substances inhibited production of cell adhesion molecules (CAMs), such as soluble intercellular adhesion molecule-1 (sICAM-1), which are also involved in inflammatory processes of the bowel.[30] These findings indicate that several anthocyanins appear to contribute synergistically to the BE's anti-inflammatory activity. The activity of anthocyanins thereby strongly depends on the aglycon structure (hydroxylation and methylation pattern) and the sugar moiety. However, it should be noted that BE also contains other flavonoids, such as proanthocyanidins, that may also contribute.

As shown by Kahle et al.,[31] anthocyanins reach the colon under physiological circumstances and are highly available in the gut. Up to 85% of the anthocyanins, depending on the sugar moiety, were determined in ileostomy bags after oral intake of anthocyanin-rich blueberries. Nevertheless, it has to be considered that they are metabolized by human gut microflora to phenolic acids and aldehydes, such as gallic acid and phloroglucinol aldehyde, within several hours.[32,33]

Additionally and in accordance with previous studies,[7,8] cy was highly unstable in cell culture medium and completely degraded after 60 min. The corresponding phenolic acid, protocatechuic acid, formed asynchronously to the degradation of cy; amounts equivalent to 5% of the initial cyanidin concentration were detected after 60 min, ca. 20% (the highest level) after 24 h, and only traces, between the LOD (162 nM) and LOQ (270 nM), of the corresponding phloroglucinol aldehyde were detected. Thus, in accordance with Forester et al.[33] we suggest that phloroglucinol aldehyde is highly unstable in cell culture media and disappears very rapidly after formation. We also detected unknown metabolites and/or degradation products by HPLC-DAD, which we assume to be dimerization products of two cy units as the reactive quinoid base is the major form of anthocyanidins in neutral media,[34] and the products were detectable at 520 nm. The stability of anthocyanidins is generally influenced by the substituents on the B-ring; additional hydroxy or methoxy groups decrease the stability of the aglycone in neutral media.[8]

In contrast, the anthocyanins were much more stable under cell culture conditions than the corresponding aglycones. One reason for their higher stability might be that the sugar moieties prevent degradation of highly unstable α-diketone intermediates[34] to the phenolic acid and aldehyde components. Furthermore, dimerization of the anthocyanidins was probably prevented by steric hindrance from the sugar moieties. Interestingly, there was no significant difference in the degradation of the investigated cyanidin-3-*O*-glycosides according to their sugar moieties, either in cell-free incubations or in incubations with cells. The similarities of their stabilities and sugar moiety-dependent anti-inflammatory activities strongly indicate that the inhibitory effects of the investigated cyanidin-3-*O*-glycosides are largely dependent on the conjugated sugars, rather than differences in their stability under cell culture conditions. Moreover, the cyanidin-3-*O*-glycosides themselves appear to be responsible for the anti-inflammatory activity, as they are almost completely stable for up to 3 h in incubations with cells. In contrast, the detected degradation products and/or metabolites of cy might contribute to its observed inhibitory effects and hence to its anti-inflammatory properties. This limited stability under cell culture conditions generally has to be considered in in vitro studies. Hence, further investigations of the effects of the observed degradation products, such as protocatechuic acid and the as yet unknown metabolites, on anti-inflammatory activities are warranted.

In summary, we identified single major anthocyanins from a bilberry (*V. myrtillus* L.) extract that modulate inflammatory genes and protein secretion in vitro and thus may act as transcription-based inhibitors of the pro-inflammatory gene expression associated with IBD. Moreover, we showed that the anti-inflammatory activity of the investigated anthocyanins is strongly dependent on their aglycone structure and the attached sugar, rather than on differences in their stability under cell culture conditions. As anthocyanins are highly available in the gut, the results suggest that these substances

might be helpful in the treatment of IBD as nutrient supplements.

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Funding

The study was supported by the Institute Danone eV. (project 2009/12).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank D. Schrenk (Department of Chemistry, Division of Food Chemistry and Toxicology, University of Kaiserslautern) and his collaborators for providing real-time PCR equipment. In addition, we kindly thank G. Erkel and M. Jung (Department of Biology, Division of Biotechnology, University of Kaiserslautern) for their support in the human cytokine antibody array studies.

ABBREVIATIONS USED

BE, bilberry extract (*Vaccinium myrtillus* L.); CM, cytokine mixture; DEPC, diethyl pyrocarbonate; GAPDH, glycerinaldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage colony-stimulating factor; GRO-α, growth-related oncogene alpha; IBD, inflammatory bowel disease; IEC, intestinal epithelial cells; IFN, interferon; IL, interleukin; I-TAC, interferon-inducible T-cell alpha chemoattractant; IP-10, IFN-γ-inducible protein-10; NF-κB, nuclear factor-kappa B; TNF, tumor necrosis factor.

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