Anthocyanins Inhibit Nuclear Factor-κB Activation in Monocytes and Reduce Plasma Concentrations of Pro-Inflammatory Mediators in Healthy Adults[1–3]

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Abstract

The transcription factor nuclear factor-κB (NF-κB) is activated by oxidative stress and pro-inflammatory stimuli and controls the expression of numerous genes involved in the inflammatory response. Dampening NF-κB activation and thereby limiting the inflammatory response have been suggested as a potential strategy to prevent chronic inflammatory diseases. In cultured monocytes, anthocyanins isolated from bilberries and black currants (Medox) efficiently suppressed LPS-induced activation of NF-κB. Furthermore, we studied the effect of anthocyanin supplementation (Medox, 300 mg/d for 3 wk) in a parallel-designed, placebo-controlled clinical trial ($n = 120$ men and women aged 40–74 y). Differences were observed in several NF-κB related inflammatory mediators in the Medox group compared to placebo. The changes in the NF-κB-controlled pro-inflammatory chemokines IL-8, ''regulated upon activation, normal T cell expressed and secreted,'' (RANTES) and IFNα (an inducer of NF-κB activation) in the Medox group (45, 15, and 40% decreases from baseline, respectively) differed from those in the placebo group (20, 0, and 15% decreases from baseline, respectively) ($P < 0.050$). Similarly, changes in IL-4 and IL-13, 2 cytokines that mediate pro-inflammatory responses and induce NF-κB activation, in the Medox group (60 and 38% decreases from baseline, respectively) tended to differ from those in the placebo group (4 and 6% decreases) ($P = 0.056$ and, $P = 0.089$, respectively). These data suggest that anthocyanin supplementation may have a role in the prevention or treatment of chronic inflammatory diseases by inhibition of NF-κB transactivation and deceased plasma concentrations of pro-inflammatory chemokines, cytokines, and inflammatory mediators. J. Nutr. 137: 1951–1954, 2007.

Introduction

Inflammation is a complex series of reactions executed by the host to prevent ongoing tissue damage and activate repair processes and defense mechanisms against infectious diseases. If prolonged, inflammation may, however, contribute to the pathogenesis of chronic diseases such as diabetes, neurodegenerative diseases, cancers, and cardiovascular disease (1–4). Dampening of inflammation may potentially retard the development of such diseases. Inflammatory injury may be mediated by reactive oxygen species (ROS)[8] or its reaction products and antioxidant therapy has been shown to prevent in vivo tissue injury during inflammation (5,6).

The transcription factor nuclear factor-κB (NF-κB) controls expression of genes involved in the inflammatory response (7,8), and is activated by oxidative stress and numerous other pro-inflammatory stimuli. Activation of NF-κB results in coordinated expression of inflammatory and innate immune genes and secretion of pro-inflammatory chemokines and cytokines (9–11). Elevated levels of pro-inflammatory cytokines and acute phase proteins have been associated with increased risk of disease and poor outcome of chronic inflammatory diseases (12,13). Thus, dampening NF-κB activation, thereby limiting the inflammatory response, has been suggested as a strategy to prevent chronic inflammatory diseases.

Anthocyanins are water-soluble red and blue flavonoid pigments. The anthocyanins are effective antioxidant compounds able to reduce lipid peroxidation and the deleterious effects of ROS in vitro. Anthocyanins have the ability to suppress cancer (14,15), cataract (16), and neurodegeneration (17) in animal models.

We hypothesized that bilberry and blackcurrant anthocyanins may inhibit NF-κB activation. To test this hypothesis, we studied whether anthocyanins inhibit LPS-induced NF-κB activation in monocytes in culture and whether inflammatory mediators were affected in healthy adult volunteers in a randomized placebo-controlled trial.

Materials and Methods

Medox anthocyanins. The Medox capsules (Medpalett Pharmaceuticals) contained purified anthocyanins isolated from bilberries (*Vaccinium myrtillus*) and blackcurrant (*Ribes nigrum*) (a mixture of 3-O-rutinosides of cyanidin and delphinidin, and 3-O-β-galactosides, 3-O-β-glucosides, and 3-O-β-arabinosides of cyanidin, peonidin, delphinidin, petunidin, and malvidin). The 3-O-β-glucosides of cyanidin and delphinidin constituted at least 40–50% of the total anthocyanins. The placebo capsules contained maltodextrin and blue color additive.

Cell culture experiments. A human monocytic cell line stably transfected with a luciferase reporter that contained 3 NF-κB binding sites (U937–3×κB-LUC cells) was used (18). Cells were grown in RPMI-1640 medium with L-glutamine (2 mmol/L), penicillin (50 kU/L), streptomycin (50 g/L), hygromycin (75 mg/L), and 10% fetal bovine serum (all from Sigma-Aldrich) at 37°C and 5% CO_2. Cells were transferred to medium with 2% fetal bovine serum and preincubated with Medox anthocyanins (final medium concentration 100 mg/L) or vehicle (dimethylsulfoxide) for 30 min and NF-κB activity was induced by LPS (1 mg/L) for 6 h. Cell viability was determined by trypan blue exclusion, with a cut-off value of 10% nonviable cells.

Luciferase activity was measured by imaging using an IVIS Imaging System 100 (Xenogen). Luminescence was integrated for 1 min at 4 min after the addition of 0.2 mg d-luciferin/mL cell medium. The number of photons emitted from each well was calculated by Living Image software (Xenogen). Gray scale images were used for reference of position.

p65 DNA binding was measured using the Trans-AM NF-κB p65 transcription factor assay kit (catalog no. 40096, Active Motif) according to the manufacturer's instructions and as previously described (19).

Subjects and intervention. The clinical study was approved by the local Regional Committee for Medical Research Ethics and all participants gave written, informed consent. Participants were recruited through advertisement in a local newspaper. All participants were healthy adults aged 40–74 y (61 women and 59 men). When entering the study, the participants completed a questionnaire related to the exclusion criteria (clinically recognized chronic diseases such as diabetes, cardiovascular disease, cancer, liver and renal disorders, chronic autoimmune disease, and chronic or acute (e.g. common cold) infections. Participants treated with statins, aspirin, or oral hormone replacement therapy were not included. We recruited only participants that did not smoke or drink alcohol daily.

This study was designed to investigate whether supplementation with Medox capsules (Medpalett Pharmaceuticals) for 3 wk affected markers of inflammation in a parallel-designed, placebo-controlled clinical study. A total of 120 participants were included and randomly assigned to the Medox or placebo group; 118 participants completed the study, 59 in each group. The Medox group was instructed to consume 2 75-mg Medox capsules 2 times/d (morning and evening), providing a total of 300 mg anthocyanins/d. The amount of anthocyanins provided daily corresponded to ~100 g of fresh bilberries (20,21). We instructed the subjects to maintain their regular diet during the intervention period. There was no assessment of the participant's habitual diet, including berry consumption. Compliance of the intervention was not assessed apart from asking the participants whether they consumed the capsules. We collected blood samples from fasting at the time of inclusion and after the intervention period; plasma and serum were stored at −70°C unless immediately analyzed.

Laboratory methods. Cytokines [IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, TNFα, IFNα, IFNγ, granolyte/macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP)1-α, MIP-1β, immuno-

protein (IP)-10, monocyte chemoattractant protein-1 (MCP-1), eotaxin, and regulated upon activation, normal T cell expressed and secreted (RANTES)] were measured in plasma by a sandwich immunoassay-based protein array system (Biosource International, kit no. LHC0009). Cytokine detection was performed according to the manufacturer's instruction, with assay diluents as blank. Calibration standards were prepared in the assay buffer. Antibody-coupled microspheres specific for the different cytokines were incubated with plasma and antigen binding was detected using a flow-based dual laser detector with real-time digital signal. The Luminex 100 IS instrument (Biosource) with the Star Station acquisition program (v2 Applied Cytometry Systems) was used to process the data. All samples were run in single wells except the standard curve points, which were run in duplicate according to the manufacturer's recommendations. For those inflammatory mediators in which changes over the intervention period were significantly different between the Medox and the placebo group, ELISA (kit nos. KHC-1301, KAC1511, and KHC 4032 for the cytokines IL-8, RANTES, and IFNα, respectively; Invitrogen) were also used.

Serum C-reactive protein (CRP) was measured using a high-sensitivity assay from Roche Diagnostics (kit no. 11972855216).

Statistical analysis. Student's *t* test and Mann-Whitney test were used for normal and non-normally distributed data, respectively, to compare the mean and median baseline values between the Medox and placebo groups at baseline. Similarly, mean and median changes during the intervention period were compared between the groups. Baseline values are presented as mean (range) or median (range) and the changes during the intervention period are presented as mean ± SD or median difference (95% CI). For the cell culture experiments, 1-way ANOVA and post hoc Bonferroni (luciferase activity assay) and Dunnett's (p65 DNA-binding assay) test were conducted. SPSS ver 13.0 was used for all statistical analysis. A *P*-value <0.050 was considered significant.

Results

LPS-induced NF-κB activation in monocytes. Cells were incubated with LPS (1 mg/L) for 6 h, which induced luciferase activity 14.2-fold (*n* = 3) compared with basal activity (data not shown). When Medox anthocyanins and LPS were coadministered to the cells, NF-κB activation was suppressed by 27.6% compared with cells incubated with LPS and vehicle only (*P* = 0.003). Medox anthocyanins also decreased the LPS-induced p65 DNA binding, another assay for NF-κB-activation, by 17.8% (*P* = 0.041).

Clinical trial. Of the 118 participants who completed the study, 61 were women and 44 were former smokers. Besides plasma IFNα, which was higher (*P* = 0.048) in the Medox group, the groups did not differ in any of the measured variables at baseline (**Table 1**).

The changes in several of the NF-κB-related inflammatory mediators in plasma differed between the groups. The changes in the NF-κB-controlled pro-inflammatory chemokines, IL-8 and RANTES, and an inducer of NF-κB activation, IFNα, in the Medox group (45, 15, and 40% decreases from baseline) differed from those in the placebo group (20, 0, and 15% decreases from baseline) (*P* < 0.050). These findings were confirmed by ELISA (**Supplemental Tables 1** and **2**).

Similarly, the decreases from baseline in the Medox group in IL-4 (60%) and IL-13 (38%), 2 cytokines that mediate pro-inflammatory responses and are inducers of NF-κB activation, tended to be greater (*P* = 0.056 and *P* = 0.089, respectively) than the changes in the placebo group (4 and 6% decreases, respectively).

The changes in plasma CRP did not differ between the groups (**Table 2**). Plasma lipids (total and HDL cholesterol and oxidized LDL) and plasma antioxidants did not differ between the groups at baseline (**Supplemental Table 3**) and changes during the intervention period did not differ between them (**Supplemental Table 4**).

TABLE 1 Baseline characteristics of the study participants[1,2]

Variable	Placebo (n = 59)	Medox (n = 59)
Age, y	61 (49–73)	61 (40–74)
Systolic BP[3], mm Hg	130 (92–180)	133 (92–181)
Diastolic BP, mm Hg	78 (54–99)	80 (57–109)
BMI, kg/m²	24 (17–34)	25 (20–35)
Circulating chemokines, cytokines, and inflammatory mediators[4]		
CRP[a], mg/L	3.2 (0.4–10.4)	3.5 (0.4–5.9)
IL-1β[a], ng/L	59 (0.1–650)	49 (0.1–2111)
IL-1Ra[a], ng/L	241 (0.1–5597)	389 (0.1–7406)
IL-2[a], ng/L	14.9 (0.1–410)	14.0 (0.1–4358)
IL-4[a], ng/L	13.9 (0.1–120)	23.2 (0.8–315)
IL-6, ng/L	20.1 (0.9–129)	20.6 (0.4–152)
IL-8 (CXCL8) [a], ng/L	3.0 (0.1–14.4)	3.2 (0.6–39.3)
IL-10[a], ng/L	20.7 (0.1–684)	24.0 (0.1–1071)
IL-12, ng/L	124 (44–362)	137 (29.6–759)
IL-13[a], ng/L	10.6 (0.1–92)	12.3 (0.1–314)
IL-17[a], ng/L	19.4 (0.1–200)	18.8 (0.1–176)
TNFα[a], ng/L	8.7 (0.1–173)	14.0 (2.0–819)
IFNα[a], ng/L	45.6 (0.1–230)	79.1* (0.1–544)
IFNγ[a], ng/L	22.9 (0.1–117)	24.6 (0.1–346)
GM-CSF[a], ng/L	70.6 (0.1–1184)	69.5 (5–2545)
MIP1-α (CCL3)[a], ng/L	24.8 (0.1–328)	24.3 (0.1–514)
MIP1-β (CCL4)[a], ng/L	201 (16–1120)	203 (16–822)
IP-10 (CXCL10)[a], ng/L	20.5 (3.7–92)	22.0 (6.1–159.2)
MCP-1 (CCL2)[a], ng/L	105 (47–243)	119 (20–380)
Eotaxin (CCL11)[a], ng/L	46.1 (15.7–106)	48.3 (18.9–126)
RANTES (CCL5), ng/L	8917 (1203–27775)	8725 (1542–20083)

[1] Values are presented as mean (range) or [a]median (range). *Significantly different from placebo, P < 0.050.
[2] 30 men and 29 women in each group.
[3] Blood pressure.
[4] All were measured in plasma except CRP, which was measured in serum.

TABLE 2 Changes from baseline in plasma NF-κB related chemokines, cytokines, and inflammatory mediators in men and women who received supplement with Medox or placebo for 3 wk[1]

Variable	Placebo (n = 59)	Medox (n = 59)
CRP[2], mg/L	0.49 (−0.39, 1.37)	0.00 (0.03, 0.968)
IL-1β, ng/L	−13.8 (−36.0, 48.6)	−12.4 (−45.1, 0.1)
IL-1Ra, ng/L	−38 (−545, −95)	−80 (−349, −75)
IL-2, ng/L	−1.8 (−15.9, 36.9)	−36.5 (−87.7, 14.7)
IL-4, ng/L	−3.6 (−13.0, −2.5)	−14.6 (−17.4, −5.8)
IL-6, ng/L	−9.3 ± 19.5	−5.9 ± 12.8
IL-8 (CXCL8), ng/L	−0.7 (−1.5, −0.2)	−1.4* (−1.9, −0.9)
IL-10, ng/L	−5.3 (−29.9, −2.7)	−6.6 (−20.0, 2.0)
IL-12, ng/L	−20.4 ± 40.7	−21.6 ± 51.3
IL-13, ng/L	−1.8 (−6.8, 0.4)	−4.7 (−10.2, −1.4)
IL-17, ng/L	−5.2 (−18.0, 0.2)	−10.0 (−18.9, −8.6)
TNFα, ng/L	−1.7 (−13.3, −1.6)	−3.9 (−29.1, 0.9)
IFNα, ng/L	−10.9 (−31.3, −5.7)	−32.3* (−54.1, −25.2)
IFNγ, ng/L	−11.4 (−24.2, −10.7)	−14.2 (−25.2, −12.9)
GM-CSF, ng/L	−15.0 (−61.2, −24.4)	−30.0 (−59.6, −11.1)
MIP1-α (CCL3), ng/L	−7.5 (−14.7, −3.9)	−11.3 (−16.4, −4.7)
MIP1-β (CCL4), ng/L	−63.8 (−155.5, −54.7)	−82.9 (−136.3, −64.3)
IP-10 (CXCL10), ng/L	1.8 (−2.6, 4.3)	0.7 (−1.2, 7.2)
MCP-1 (CCL2), ng/L	−15.0 (−28.4, −10.1)	−23.9 (−33.9, −9.8)
Eotaxin (CCL11), ng/L	−7.7 (−11.7, −2.0)	−6.1 (−7.0, 2.0)
RANTES (CCL5), ng/L	4 ± 2944	−1057* ± 2700

[1] Values are mean ± SD or median difference (95% CI). * Significantly different from placebo, P < 0.050.
[2] Measured in serum.

Discussion

The transcription factor NF-κB is essential in orchestrating the inflammatory responses to a wide range of insults (10). Several NF-κB related pro-inflammatory chemokines, cytokines, and mediators of inflammatory responses decreased in plasma of healthy adult volunteers after supplementation with Medox anthocyanins for 3 wk in the parallel-designed, placebo-controlled clinical trial. This suggests that anthocyanin supplementation may mediate an inhibition of NF-κB activation in vivo (10,22–26). These findings were supported by the observation that Medox anthocyanins suppressed LPS-induced NF-κB activation, as measured by decreased transactivation of the luciferase reporter and p65 DNA binding in human monocytes.

A recent clinical study using anthocyanin-rich sweet cherries supports our observations; CRP and RANTES decreased after 4 wk of intervention (27). The clinical trial reported herein represents, to our knowledge, the first human intervention trial in which effects of pure anthocyanins on inflammatory mediators have been studied.

Several previous studies have reported that anthocyanins modulate the inflammatory responses in cell cultures and animal models. Anthocyanins inhibited secretion of pro-inflammatory cytokines such as IL-8, MCP-1, IL-1β, cytokine-induced neutrophil chemoattractant-1, IL-6, and TNFα in cellular and animal models after inflammatory insults (28–33). Similarly, anthocyanins suppressed induced secretion of several molecules related to inflammatory modulation, specifically vascular endo-

thelial growth factor and intracellular adhesion molecule-1 in cellular models (33–35). In several studies, the suppression of pro-inflammatory chemokines, growth factors, and adhesion molecules were associated with an inhibition of NF-κB activation (28,29,34). The mechanisms whereby anthocyanins inhibit NF-κB activation are not fully understood. One possible mechanism is that anthocyanins, their breakdown products, or metabolites serve as redox buffers capable of suppressing oxidative stress and thereby dampen the inflammatory response by direct ROS scavenging. These events may eventually be followed by decreased secretion of pro-inflammatory signaling molecules and mediators, as observed in this study.

It is plausible that anthocyanins are able to inhibit NF-κB activation in vivo. If so, dampening NF-κB activation and limiting the inflammatory responses may represent prevention and treatment strategy for chronic inflammatory diseases.

Whereas intact anthocyanins are poorly absorbed (36), several metabolites are absorbed more efficiently (37,38). It has previously been observed that anthocyanins or metabolites do exhibit antioxidant activity in vivo in experimental models of healthy animals exposed to severe oxidative stress (39–45).

In conclusion, supplementation with Medox anthocyanins to healthy adults for 3 wk decreased the plasma concentrations of several NF-κB-regulated pro-inflammatory chemokines and immunoregulatory cytokines. Direct inhibition of LPS-induced NF-κB transactivation by anthocyanins was observed in human monocytes. The potential beneficial effect of decreased plasma concentrations of several inflammatory mediators in healthy adult volunteers after anthocyanin supplementation suggests that anthocyanins possess anti-inflammatory effects. Dampening NF-κB activation and limiting inflammatory responses by anthocyanin supplements should therefore be tested further as a strategy for prevention and treatment of chronic inflammatory diseases.

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