Effects of blackcurrant-based juice on atherosclerosis-related biomarkers in cultured macrophages and in human subjects after consumption of a high-energy meal

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Abstract

Regular consumption of fruit and vegetables may be associated with decreased CVD risk. In the present study, we investigated the effects of blackcurrant (BC) juice, rich in polyphenols and ascorbic acid, on oxidative and inflammatory biomarkers in cultured macrophages *in vitro* and in human subjects with an atherosclerosis-prone phenotype (after consumption of a high-energy meal). In cultured macrophages (RAW264.7), BC treatment significantly inhibited lipopolysaccharide-induced inflammation as indicated by lower mRNA levels of TNF-α, IL-1β and inducible NO synthase (iNOS) and lower nuclear p65 levels indicating decreased NF-κB activity. iNOS protein levels were lower and haem oxygenase 1 levels higher in BC-treated cells when compared with untreated controls. Subjects given a high-energy meal had elevated serum glucose and insulin levels with no significant difference between the BC-based juice and placebo treatment groups. TAG following meal ingestion tended to be attenuated after the BC treatment. Plasma ascorbic acid and radical-scavenging capacity were decreased following placebo meal consumption; however, BC significantly elevated both parameters compared with baseline and placebo ingestion. Plasma oxidised LDL, α-tocopherol and paraoxonase activity were unchanged in both treatment groups. Furthermore, production of TNF-α and IL-1β was not significantly changed by BC meal consumption. The present results suggest potential antioxidative and anti-inflammatory properties of BC *in vitro* in cultured macrophages. Although the observations were not directly transferable to a postprandial *in vivo* situation, the present results show that BC juice consumption may improve postprandial antioxidant status as indicated by higher ascorbic acid levels and free radical-scavenging capacity in plasma.

Key words: Polyphenolic compounds: Ascorbic acid: Pro-inflammatory cytokines: Postprandial state

CVD is the leading cause of premature death in Western populations. Oxidative modification of LDL is considered to be a major trigger of atherosclerosis[1]. Furthermore, there is increasing experimental evidence that inflammatory processes play a central role in the aetiology and progression of atherosclerosis[2]. Atherosclerotic modifications of the artery wall are dependent on both lifestyle and nutrition-related factors. Epidemiological and prospective studies support an inverse relationship between CVD and fruit and vegetable consumption[3–6]. Despite the scarcity of clinical studies investigating the underlying mechanisms, beneficial effects of fruit and vegetables have often been attributed to the bioactive compounds within them, such as antioxidant vitamins and polyphenols. Studies in cultured cells and laboratory mice have demonstrated that the compounds in fruit and vegetables have diverse actions including antioxidant, anti-inflammatory and gene-regulatory properties[7–11]. Recent studies have suggested that fruit and vegetables potentially modify postprandial progression of oxidation and inflammation as evidenced by oxidative and inflammatory biomarkers. The postprandial state, particularly after a meal rich in fat and carbohydrates, is associated with a sharp increase in blood glucose, TAG, oxidative stress and inflammation[12]. Interestingly, the concomitant intake of orange juice and strawberries

has recently been shown to attenuate postprandial oxidative and inflammatory stress[13,14].

Blackcurrant (BC) and other berry juices are particularly rich in vitamin C and polyphenols including anthocyanins[15,16]. In the present study, we investigated the effects of BC on oxidative and inflammatory biomarkers in cultured macrophages (as a model for postprandial increased inflammation) and in human subjects with an atherosclerosis-prone phenotype after consumption of a high-energy meal.

Materials and methods

In vitro analyses of fruit juice concentrates

Fruit juice concentrates (BC, red grape, cherry and raspberry) were provided by Schwartauer Werke (Bad Schwartau, Germany). Fruit juices were diluted (1:4, w/w) with methanol and centrifuged (11 000 g, 10 min, at 4°C). Supernatants were used for the analysis of their *in vitro* antioxidative capacity and in cell-culture experiments. Final methanol concentration for all cell-culture experiments was <0·1% (v/v).

Radical-scavenging capacity was measured using the oxygen radical absorbance capacity (ORAC) assay[17]. In brief, peroxyl radicals were generated by 2,2′-azobis(2-amidino-propan), incubated with sodium fluorescein, and fluorescence measured at 485/520 nm (excitation/emission) every 1 min for 50 min. Trolox served as reference and ORAC values were calculated by area under the curve of time-dependent changes in fluorescence. Results were related to 1 μmol/l of Trolox and are expressed as Trolox equivalents.

For quantification of ascorbic acid, fruit juice concentrates were diluted (1:2, w/w) in 1·5% *meta*-phosphoric acid and then incubated with 2,5-diphenyl-3-thiazolyltetrazolium chloride. Absorbance was measured at 540 nm. Ascorbic acid concentrations were calculated applying an external curve. Total polyphenolic content of fruit juices was measured using Folin−Ciocalteu's reagent, measuring absorbance at 720 nm. Gallic acid was used as a reference.

Anthocyanin analyses were carried out using an Agilent Technologies 1200 Series liquid chromatograph with a quaternary pump and photodiode array detector (DAD). The HPLC system was equipped with a Phenomenex Aqua C18 column (5 μm; 200 Å; 4·6 × 150 mm), which was set thermostatically at 35°C. Solvents used were aqueous 4·5% formic acid (solvent A) and HPLC-grade acetonitrile (solvent B) at a flow rate of 0·5 ml/min. Starting isocratically with 10% B up to 20 min, the gradient was 13% B from 20 to 35 min, 15% B from 35 to 55 min, 35% B from 55 to 65 min and 10% B from 65 to 70 min. Detection wavelengths were 280, 360 and 520 nm. Analyses were carried out in triplicate. Total anthocyanins in the juice were quantified at 520 nm by integrating all the peaks. The concentration of 3-monoglucosides of delphinidin, cyanidin and malvidin in the samples was determined using commercially available standards.

Cell-culture experiments

Murine macrophages (RAW264.7) were purchased from DSMZ (Braunschweig, Germany). Cell-culture medium and supplements were from PAA (Coelbe, Germany). Cells were grown in a humidified atmosphere at 37°C and 5% CO_2. RAW264.7 cells were incubated with test compounds (final concentration of fruit juices in cell-culture medium was 0·2 mg/ml) for 4 h followed by stimulation with 10 ng/ml of lipopolysaccharide (LPS; Sigma, Hamburg, Germany). Cells were harvested at indicated time points for the isolation of RNA and protein and for the quantification of cellular cholesterol concentration.

mRNA expression

For total RNA isolation, cells were lysed using TRIsure reagent (Bioline, Luckenwalde, Germany). Organic and aqueous phases were separated after chloroform addition and centrifugation, and RNA was precipitated with isopropanol. Total RNA concentration was quantified by measuring absorbance at 260 nm and RNA purity was controlled by calculating the ratio of 260 nm/280 nm on a spectrophotometer (Beckman Coulter, Krefeld, Germany). One-step real-time RT-PCR analyses were performed, as described previously[18], using the SensiMix™ One-Step Kit (Quantance, Bioline) on a Rotor Gene 6000 thermocycler (Corbett Research, Sydney, Australia). Primers (Table 1) were designed by Primer3 Input software (version 0.4.0). Relative mRNA levels of target genes were related to housekeeping gene expression.

Protein levels

For Western blotting, whole-cell homogenates and nuclear extracts were prepared as described previously[19]. Proteins were separated by SDS−PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Munich, Germany). Target proteins were identified using respective primary and secondary antibodies (anti-haem oxygenase 1 (HO-1; Biomol, Hamburg, Germany), anti-inducible NO synthase (iNOS; Stressgen, Enzo Life Sciences, Loerrach, Germany), anti-α-tubulin, anti-p65, anti-actin, anti-TATA box binding protein

Table 1. Sequences of primers used for one-step real-time RT-PCR

	Forward	Reverse
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
iNOS	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
CD36	CAAAACGACTGCAGGTCAAC	CCAATGGTCCCAGTCTCATT
GAPDH	CCGCATCTTCTTGTGCAGT	GGCAACAATCTCCACTTTGC

iNOS, inducible NO synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(TBP) and all secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany). Protein bands were visualised using the Pierce® ECL Western Blotting Substrate kit (Thermo Scientific, Schwerte, Germany).

Cellular cholesterol

For the determination of cellular cholesterol, cells were homogenised in potassium phosphate buffer and incubated with 20 mmol/l of sodium cholate in potassium phosphate buffer with 1% Triton X-100. Total cholesterol was determined by enzymatic conversion of cholesterol generating H_2O_2. Peroxidation of 4-hydroxyphenylacetate by H_2O_2 results in the formation of fluorescent 4-hydroxyphenylacetate-dimers (2,2′-dihydroxy-5,5′-dicarboxy-methyl-biphenyl). Fluorescence measured at 360/465 nm (excitation/emission) was directly proportional to total cholesterol content. Cellular cholesterol was quantified using an external cholesterol standard curve.

Study subjects and study design

Male study subjects were recruited from the local community according to the following inclusion criteria: BMI 25–35 kg/m², total cholesterol > 5·2 mmol/l and LDL-cholesterol > 3·6 mmol/l. Exclusion criteria were diagnosed diabetes mellitus, history of cardiovascular events, regular intake of statins, occurrence of any chronic disease and smoking. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and the study protocol was approved by the ethics committee of the Medical Association, Bad Segeberg, Germany. Written informed consent was obtained from all subjects.

The study was performed using a single-blind, placebo-controlled, cross-over design. Each participant was given a high-energy meal in combination with (1) a BC-based fruit beverage (15% BC purée, 9% raspberry purée, 7% cherry purée and 39% red grape juice) or (2) a placebo beverage. Both beverages contained similar amounts of banana purée resulting in similar viscosity and texture. The high-energy meal served as a stimulus for postprandial changes in atherosclerosis-related biomarkers. The meal was composed of 200 g cream (30% fat), 75 g sucrose and 250 g BC or placebo beverage (Table 2). The BC and placebo beverages were identical in energy, sucrose, fructose and glucose, and adjusted for dietary fibre content. The BC and placebo beverages were different in ascorbic acid (122 *v.* <1 mg/ 250 g) and total polyphenol content (617 *v.* 41 mg gallic acid equivalents/250 g). Overnight fasting blood samples were taken before ingestion of the test meal (assigned as 0 min). After consumption of the respective test meals, venous blood was taken at consecutive time points (at 15, 30, 45, 60, 90, 120, 150, 180, 210 and 240 min following the test meal intake). Each participant was given the BC and placebo test meals at intervals of 6–7 d.

Blood analyses

Blood samples were collected in heparinised syringes, and plasma, separated by centrifugation, was stored at −80°C

Table 2. Ingredients of the test meals composed of cream and sugar in combination with a blackcurrant (BC)-based or placebo beverage (placebo)

	BC	Placebo
Energy		
kcal	1030	1029
kJ	4310	4305
Fat (g)	63·8	63·8
Protein (g)	6·1	6·0
Carbohydrates (g)	107·8	107·6
Dietary fibre (g)	2·5	1·8
Ascorbic acid (mg)	122·3	0·3
Polyphenolic compounds (mg)	617	41
Anthocyanins (mg)	11·2	nd
Delphidin-3-glucoside	7·5	–
Cyanidin-3-glucoside	2·9	–
Malvidin-3-glucoside	0·8	–
ORAC value (mmol/l TE)	5·2	0·1

nd, Not detected; ORAC, oxygen radical absorbance capacity; TE, Trolox equivalents.

until analysis. Plasma TAG and cholesterol content was analysed using standard methods by a local service biochemical laboratory (Dres. Ballies, Kiel, Germany). Only one subject was excluded from TAG analyses due to his especially high basal TAG level (10·7 mmol/l). Radical scavenging capacity (ORAC) of plasma was measured as described above. Plasma IL-6 concentration was measured using a quantitative sandwich enzyme immunoassay (R&D, Wiesbaden, Germany), according to the manufacturer's protocols.

Ascorbic acid and α-tocopherol

Plasma ascorbic acid was measured following precipitation with perchloric acid by HPLC using a LiChrospher 100 column (125 × 4 mm, RP-18, 5 µm; Merck, Darmstadt, Germany). Ascorbic acid concentrations were analysed using a Jasco HPLC system (Jasco Corporation, Gross-Umstadt, Germany) with a UV detector set on 243 nm and NaH_2PO_4 (45 mmol/l) as the mobile phase. An external standard curve was used. Plasma α-tocopherol was measured using a Waters spherisorb ODS-2 column (100 × 4·6 mm, 3 µm) and methanol–water (98:2, w/w) as the mobile phase. The concentration of α-tocopherol was identified by fluorescence at 290/350 nm (excitation/emission) and calculated using an external α-tocopherol standard curve.

Anthocyanins and anthocyanin metabolites

Plasma concentrations of anthocyanins and anthocyanin metabolites were determined using an Agilent 1100 series liquid chromatograph/mass-selective detector equipped with a quadrupole (G1946D) mass spectrometer (Agilent Technologies, Waldrom, Germany). After protein precipitation from plasma, anthocyanins and their metabolites were extracted with cold methanol and concentrated using a Speed-Vac. The samples were then taken up in 0·2 ml water–methanol (1:1, v/v), vortexed and passed through a 4 mm polyvinylidene-difluoride 0·2 µm syringe filter into vials for HPLC analysis. The liquid chromatographic system consisted of

a quaternary pump (G1311A), online vacuum degasser, autosampler (G1313A) and thermostatic column compartment, connected in line to a DAD (G1315B) before the mass spectrometer. Separation was achieved with an ORBAX Eclipse XDB-C18, 4·6× 150 mm, 5 μm (Agilent Technologies). Elution was performed with a gradient between 2·5 % acetic acid in Milli-Q water (solution A), a mixture of 2·5 % acetic acid in Milli-Q water–acetonitrile (90:10, solution B) and pure acetonitrile (solution C) at a flow rate of 0·5 ml/min and an injection volume of 20 μl and an elution programme consisting of the following: from 100 % A to 100 % B in 3 min, from 100 % to 93 % B in 5 min, from 7 % to 10 % C in B in 7 min, from 10 % to 15 % C in 5 min, from 15 % to 50 % C in 5 min and isocratic 50 % C and B for another 5 min. Electrospray ionisation in the positive ionisation mode was used. The electrospray capillary voltage was set to 2500 V, with a nebulising gas flow rate of 12 litres/min and a drying gas temperature of 150°C. MS analyses were recorded in the single ion monitoring (SIM) mode at 493, 449 and 465 m/z for malvidin-3-glucoside, cyanidin-3-glucoside and delphinidin-3-glucoside, at 331, 287 and 303 for their respective aglycons, malvidin, cyanidin and delphinidin and at 507, 463 and 479 for their corresponding glucuronides. Full MS analyses were also recorded and from them the following ions (m/z) were extracted as post-analysis SIM in the positive mode at: 127, 171, 155, 169, 285, 139, 225 and 165 corresponding to 1,3,5-trihydroxybenzene (phloroglucinol), gallic acid, protocatechuic acid, vanillic acid, caffeic acid, hydroxybenzoic acid, sinapic acid and coumaric acid.

Paraoxonase

Paraoxonase (PON) activity in plasma was determined spectrophotometrically (Beckman Coulter) using phenylacetate as the substrate as described previously[20,21]. PON activity was related to HDL-concentration in plasma.

TNF-α and IL-1β

For quantification of TNF-α and IL-1β, whole blood was diluted (1:10, w/w) in RPMI-1640 with 10 % fetal bovine serum and cultured at standard conditions (see above). Whole-blood cultures were stimulated with LPS (100 ng/ml) and cell-culture supernatants were collected after 24 h. Cytokine concentrations were determined using respective quantitative sandwich enzyme immunoassays (R&D), according to the manufacturer's protocols.

Statistical analysis

Data are presented as means with their standard errors. mRNA expression results from cell-culture experiments were analysed by the t test for independent samples and, in the absence of normal distribution, by the Mann–Whitney U test. Since Western blotting is only a semi-quantitative measurement of protein levels, we did not apply statistical tests as far as the densitometric analysis of the Western blots is concerned. Data from the human study were analysed using paired t tests (comparing results from the BC $v.$ placebo treatment or baseline $v.$ postprandial changes). Differences were considered significant when $P < 0.05$. All statistical calculations were performed using SPSS version 15.0 (IBM, Ehningen, Germany).

Results

In vitro analysis and cell-culture experiments

Radical-scavenging capacity and concentrations of polyphenolic compounds and ascorbic acid were measured in juice concentrates of BC, raspberry, cherry and red grape. BC and raspberry exhibited highest and second highest ORAC values, respectively. Furthermore, BC contained the highest levels of total polyphenols and ascorbic acid compared with raspberry, cherry and red grape (Table 3). Due to its relatively high levels of antioxidative compounds, BC was selected to systematically investigate its potential anti-inflammatory and anti-atherogenic effects in isolated cells.

At the mRNA level, pre-incubation of macrophages with BC significantly inhibited ($P < 0.001$) LPS-induced expression of pro-inflammatory molecules such as TNF-α, IL-1β and iNOS compared with cells treated only with LPS (Fig. 1(a)–(c)). Relative mRNA levels of the scavenger receptor CD36 were likewise reduced in BC-pre-treated cells compared with LPS (Fig. 1(d)). Since LPS stimulates inflammatory genes via NF-κB transactivation, nuclear levels of the NF-κB subunit p65 were examined in the absence and presence of BC. Incubation with BC significantly reduced LPS-induced nuclear translocation of p65 compared with control LPS-stimulated cells (Fig. 2). iNOS protein was up-regulated by concomitant LPS and interferon-γ stimulation in our cells. Pre-incubation with BC almost completely abolished this iNOS up-regulation (Fig. 2). Furthermore, protein levels of the antioxidative haem oxygenase 1 were higher in BC-treated cells compared with untreated control cells.

Since accumulation of cholesterol in macrophages triggers foam-cell formation, cellular cholesterol levels were measured in the absence (43 (SEM 2) ng/mg) and presence (60 (SEM 4) ng/mg) of LPS. Under the conditions investigated, the BC treatment counteracted the LPS-induced increase in cellular cholesterol levels (37 (SEM 1) ng/mg).

Human study

A total of eleven male participants (37·4 (SEM 1·9) years, 32·1 (SEM 1·2) kg/m²) were included in the study (baseline

Table 3. Data of *in vitro* analyses of different fruit juice concentrates

(Mean values with their standard errors)

|	ORAC value (mmol/l TE)		Total polyphenol content (g/l)		Ascorbic acid (g/l)	
	Mean	SEM	Mean	SEM	Mean	SEM
Blackcurrant	35·4	1·1	95·9	3·4	41·8	0·5
Raspberry	11·8	1·4	42·1	0·4	5·3	0·2
Cherry	6·1	0·7	26·8	0·2	3·6	0·1
Red grape	5·8	0·8	8·9	0·1	3·3	0·1
Banana purée	0·4	0·1	2·0	0·1	1·4	0·1

TE, Trolox equivalents.
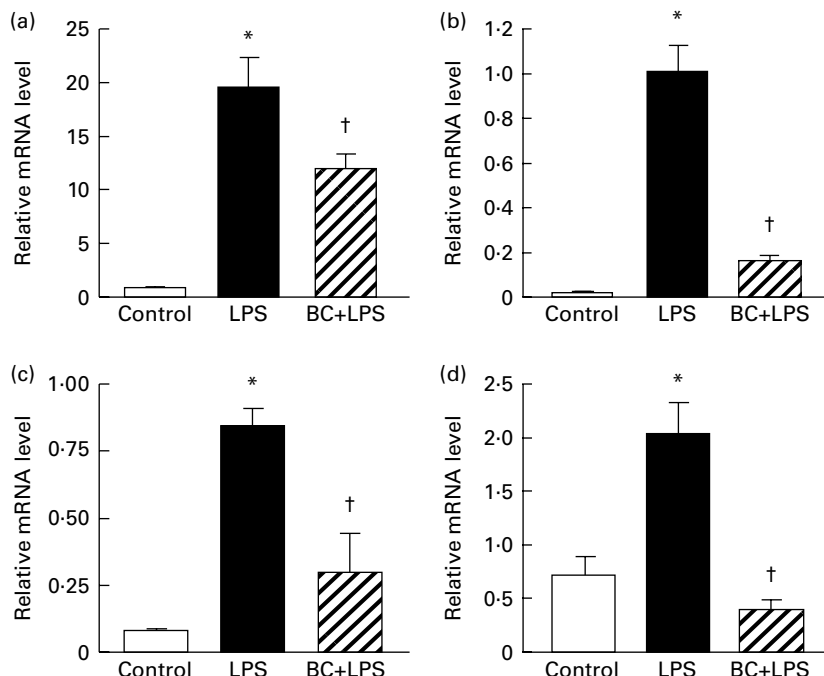
Fig. 1. Relative mRNA levels of inflammatory genes in RAW264.7 macrophages following incubation with blackcurrant (BC) fruit juice concentrate. Cells were pre-incubated with BC for 4 h and subsequently stimulated with lipopolysaccharide (LPS) for 1 h ((a) TNF-α) or 6 h ((b) IL-1β, (c) inducible NO synthase (iNOS) and (d) CD36). Untreated cells were used as control. Total RNA was isolated and relative mRNA levels were determined using real-time RT-PCR. Values are means, with standard errors represented by vertical bars (n 4–8). * Mean values were significantly different between the untreated (control) and LPS-treated cells (LPS; $P < 0.05$). † Mean values were significantly different between the BC pre-incubated LPS-treated cells (BC + LPS) and LPS ($P < 0.05$).

characteristics are summarised in Table 4). Plasma concentrations of cyanidin, delphinidin and malvidin were analysed after consumption of the BC-based test meal. However, neither anthocyanins in their glycosylated forms nor aglycones and glucuronides were detectable in plasma at any of the postprandial time points investigated. Plasma levels of other suggested anthocyanin metabolites were measured, including protocatechuic acid[22], *P*-hydroxybenzoic acid and vanillic acid[23], and also gut-derived metabolites such as 1,3,5-trihydroxybenzene[24]. Plasma levels of 1,3,5-trihydroxybenzene were increased from 71 (SEM 19) nmol/l to 187 (SEM 41) nmol/l 30 min after consumption of the BC meal, decreasing to basal levels after 150 min.

Postprandial changes in plasma glucose and insulin are shown in Fig. 3. Both parameters were increased at 15 and 30 min after ingestion of the test meals, although no
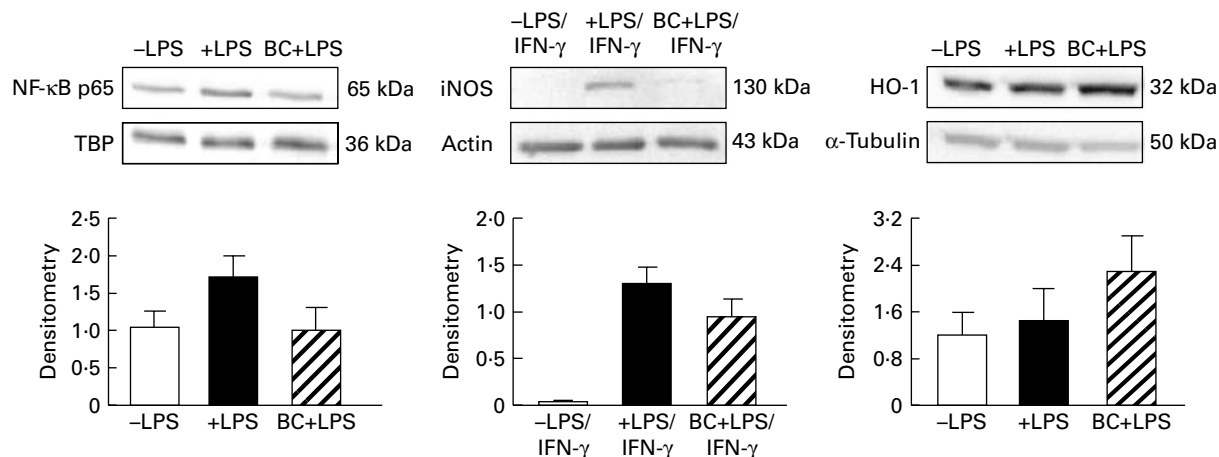
Fig. 2. Protein levels of NF-κB p65, haem oxygenase 1 (HO-1) and inducible NO synthase (iNOS) in RAW264.7 macrophages following incubation with blackcurrant (BC) fruit juice concentrate. Cells were pre-incubated with BC for 24 h and subsequently stimulated with lipopolysaccharide (LPS) for 4 h (p65), 24 h (HO-1) or LPS in combination with interferon-γ (IFN-γ) for 24 h (iNOS). Nuclear extracts (p65) or whole-cell lysates (iNOS and HO-1) were used for Western blotting analyses and one representative blot is shown, respectively. Densitometry was applied to relate target protein expression to loading controls. Values are means, with standard errors represented by vertical bars (n 3–4).

Table 4. Baseline characteristics and fasting blood parameters of male volunteers (n 11) participating in the human study

(Mean values with their standard errors)

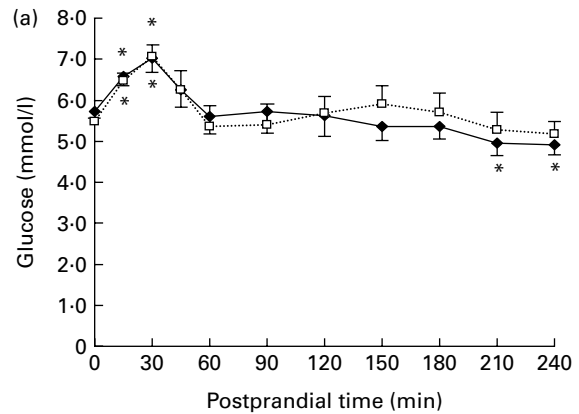|	Mean	SEM
Age (years)	37·4	1·9
BMI (kg/m^2)	32·1	1·2
Total cholesterol (mmol/l)	6·04	0·37
LDL-cholesterol (mmol/l)	4·04	0·30
HDL-cholesterol (mmol/l)	1·09	0·08
TAG (mmol/l)	2·25	0·59
Glucose (mmol/l)	5·61	0·13
Insulin (pmol/l)	84·2	9·1

differences were observed in peak concentrations between the BC and placebo treatments. At 30 min after both test meals, glucose and insulin levels declined. The rate of insulin decline tended to be faster after the placebo meal compared with the BC meal (t_{min} = 65 v. 131 min, P=0·082).

Plasma concentrations of TAG increased in response to the high-energy test meals, with maximal levels occurring at 171 and 144 min after both BC and placebo meals (Fig. 4(a)). After 240 min, plasma TAG levels were still significantly increased compared with baseline (P<0·001 and P=0·007 for BC and placebo, respectively). No significant differences in postprandial TAG levels were observed between the BC and placebo groups, although there was a trend towards lower levels in the BC group (P=0·059 at 60 min).

Postprandial changes in plasma radical-scavenging capacity (ORAC) and ascorbic acid are summarised in Fig. 4(b) and (c), respectively. After placebo consumption, ORAC values decreased over time from 2·4 at 0 min to 1·6 mmol/l Trolox equivalents at 240 min, indicating a reduction in anti-oxidant capacity in response to the high-energy meal. In contrast, ORAC values declined in the first 60 min after the BC meal but then rose again to the initial level. ORAC values of the BC group were significantly higher compared with the placebo group after 90 and 120 min (P<0·040 and P<0·002). Also, plasma ascorbic acid concentrations were significantly elevated 120 min after meal consumption in BC-treated subjects compared with the placebo group (P≤0·004); this elevation persisted until the last reading at 240 min. Ascorbic acid levels increased from 39 to 53 mmol/l after consumption of the BC-rich juice.

Further biomarkers were measured postprandially (0 v. 240 min) in plasma including total cholesterol, LDL- and HDL-cholesterol, oxidised LDL (oxLDL), α-tocopherol and PON activity (Table 5). Total cholesterol, LDL and HDL levels were significantly lower following test meal consumption, though no differences were observed between the BC and placebo treatments.

Postprandial levels of oxLDL, α-tocopherol and PON activity were not significantly different from basal levels and between the BC and placebo treatment groups. The production of TNF-α and IL-1β in response to LPS (measured in *ex vivo* whole-blood cultures) was significantly decreased in response to the placebo test meal. Interestingly, after consumption of the BC test meal, this decrease was not
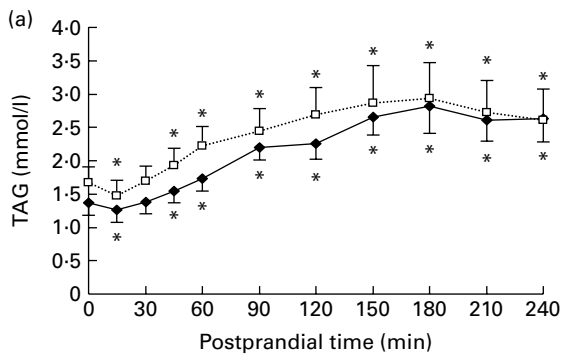

(a)

	Blackcurrant		Placebo		
	Mean	SEM	Mean	SEM	P
c_{max} (mmol/l)	7·5	0·1	7·3	0·1	0·923
t_{max} (min)	55	4	40	3	0·365
c_{min} (mmol/l)	4·6	0·1	4·5	0·1	0·760
t_{min} (min)	127	7	131	7	0·896
$AUC_{(0-120)}$ (mmol/l × min)	722	46	706	61	0·421
$AUC_{(0-240)}$ (mmol/l × min)	1351	76	1375	88	0·558

(b)
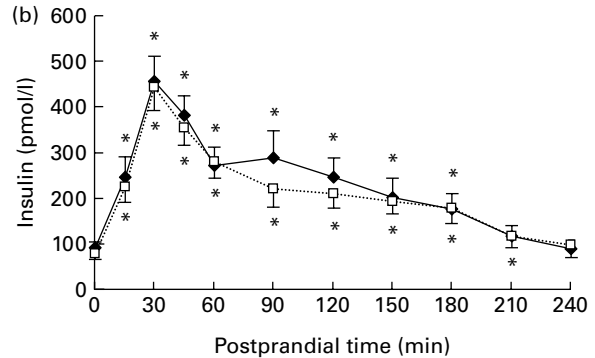
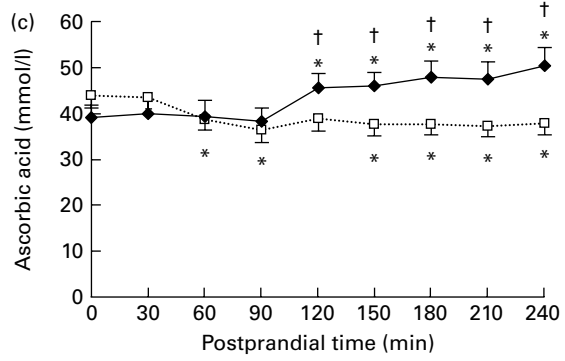|	Blackcurrant		Placebo		
	Mean	SEM	Mean	SEM	P
c_{max} (mmol/l)	509	49	478	45	0·497
t_{max} (min)	40	2	38	1	0·839
c_{min} (mmol/l)	74	4	67	3	0·263
t_{min} (min)	131	11	65	10	0·082
$AUC_{(0-120)}$ (mmol/l × min)	35	5	32	4	0·211
$AUC_{(0-240)}$ (mmol/l × min)	55	7	51	6	0·305

Fig. 3. Postprandial progression of (a) glucose and (b) insulin levels in the serum of male volunteers after ingestion of a high-energy meal in combination with a blackcurrant (BC, ◆)-based juice or placebo (⋯□⋯) beverage. Values are means of maximal and minimal plasma concentrations (c_{max}, c_{min}), respective lag time (t_{max}, t_{min}) and area under the curve (AUC_{0-120}, AUC_{0-240}), with standard errors represented by vertical bars (n 11). * Mean values were significantly different (P<0·05; paired t tests) for postprandial changes compared with baseline (0 min).

Fig. 4. Postprandial progression of (a) TAG, (b) oxygen radical-scavenging capacity (ORAC) and (c) ascorbic acid in the plasma of male volunteers after ingestion of a high-energy meal in combination with a blackcurrant (BC, ◆-)-based or placebo (··· □ ···) beverage. Values are means of maximal and minimal plasma concentrations (c_{max}, c_{min}), respective lag time (t_{max}, t_{min}) and area under the curve (AUC_{0-120}, AUC_{0-240}), with standard errors represented by vertical bars (n 10–11). * Mean values were significantly different ($P < 0.05$; paired *t* tests) for postprandial changes compared with baseline (0 min). † Mean values were significantly different ($P < 0.05$; paired *t* tests) between the BC v. placebo treatment. TE, Trolox equivalents.

(a)

	Blackcurrant		Placebo		
	Mean	SEM	Mean	SEM	P
c_{max} (mmol/l)	3·3	0·3	3·2	0·5	0·844
t_{max} (min)	171	5	144	6	0·310
c_{min} (mmol/l)	1·3	0·2	1·5	0·3	0·204
t_{min} (min)	14	2	18	2	0·177
$AUC_{(0-120)}$ (mmol/l × min)	183	19	218	47	0·136
$AUC_{(0-240)}$ (mmol/l × min)	528	53	588	92	0·351

(b)
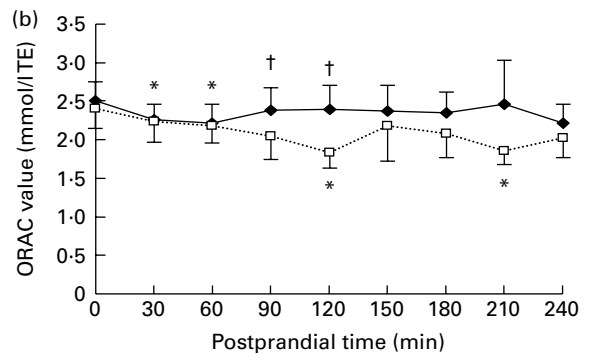
	Blackcurrant		Placebo		
	Mean	SEM	Mean	SEM	P
c_{max} (mmol/l)	3·0	0·5	2·7	0·4	0·205
t_{max} (min)	98	30	933	31	0·872
c_{min} (mmol/l)	1·9†	0·2	1·6	0·2	0·012
t_{min} (min)	136	24	142	24	0·863
$AUC_{(0-120)}$ (mmol/l × min)	279†	27	257	25	0·006
$AUC_{(0-240)}$ (mmol/l × min)	563†	63	499	54	0·008

(c)

	Blackcurrant		Placebo		
	Mean	SEM	Mean	SEM	P
c_{max} (mmol/l)	53	4	46	3	0·199
t_{max} (min)	173†	23	23	9	0·000
c_{min} (mmol/l)	33	2	35	3	0·399
t_{min} (min)	65†	20	18	2	0·023
$AUC_{(0-120)}$ (mmol/l × min)	4802	283	4798	332	0·988
$AUC_{(0-240)}$ (mmol/l × min)	10484†	705	9329	575	0·037

significant, implying that the postprandial reduction of TNF-α and IL-1β secretion was attenuated compared with placebo. Postprandial TNF-α levels were significantly lower in the placebo than in the BC treatment group ($P < 0.001$). Furthermore, circulating IL-6 was measured in plasma at 0, 120 and 240 min following ingestion of the test meals. Interestingly,

postprandial IL-6 mean values were increased in the BC group from 2·74 (SEM 0·43) pg/ml to 3·24 (SEM 0·41) pg/ml ($P = 0.385$) and 5·03 (SEM 0·81) pg/ml ($P = 0.009$) after 120 and 240 min, respectively, whereas mean values in the placebo group were increased after 120 min from 3·58 (SEM 0·56) to 4·48 (SEM 0·85) pg/ml ($P = 0.330$), but then decreased

Table 5. Postprandial changes in total cholesterol (TC), LDL- and HDL-cholesterol, oxidised LDL (oxLDL), α-tocopherol, paraoxonase (PON) activity, TNF-α and IL-1β of male volunteers after ingestion of a high-energy meal in combination with a blackcurrant (BC)-based or placebo beverage (placebo)
(Mean values with their standard errors)

| | BC | | | | | Placebo | | | | | P (treatment)* | |
| | 0 min | | 240 min | | | 0 min | | 240 min | | | | |
	Mean	SEM	Mean	SEM	P (time)†	Mean	SEM	Mean	SEM	P (time)†	0 min	240 min
TC (mmol/l)‡	5·99	0·31	5·76†	0·27	0·012	6·08	0·33	5·78†	0·28	0·009	0·569	0·856
LDL (mmol/l)‡	4·02	0·25	3·77†	0·23	0·000	4·07	0·25	3·76†	0·21	0·000	0·780	0·969
HDL (mmol/l)‡	1·08	0·08	1·00†	0·07	0·016	1·10	0·07	0·99†	0·06	0·003	0·601	0·683
oxLDL (U/ml)‡	72·9	3·6	71·4	3·7	0·625	75·3	2·5	70·9	3·5	0·140	0·370	0·911
α-Tocopherol (μmol/l)‡	35·1	7·4	27·0	2·3	0·321	33·2	4·1	29·7	3·1	0·300	0·758	0·295
PON (kU/mmol HDL)‡	95·8	10·6	106·2	13·2	0·254	98·2	8·7	103·2	10·6	0·402	0·192	0·654
TNF-α (ng/ml)§	0·81	0·15	0·73	0·10	0·238	0·68	0·10	0·58*†	0·08	0·006	0·268	0·000
IL-1β (ng/ml)§	1·25	0·09	1·17	0·09	0·076	1·24	0·08	1·14†	0·09	0·047	0·842	0·357

* Mean values of BC were significantly different from those of placebo treatment ($P<0.05$; paired t test).
† Mean values at 0 min were significantly different from those of 240 min ($P<0.05$; paired t test).
‡ Concentrations of TC, LDL-cholesterol, HDL-cholesterol, oxLDL, α-tocopherol and PON activity were determined in plasma.
§ TNF-α and IL-1β production was measured in *ex vivo* stimulated whole-blood cultures before (0 min) and after (240 min) the test meal.

after 240 min to 3·92 (SEM 0·84) pg/ml ($P=0.285$). Plasma IL-6 levels were not significantly different when comparing the treatment groups (Fig. 5).

Discussion

Fruit and vegetables may reduce the risk of CVD partly due to their high content of polyphenolic and other bioactive compounds. However, experimental evidence is limited, especially concerning the potential health benefits of fruits and fruit juices in the postprandial state. Postprandial increases in insulin, glucose and particularly TAG may induce oxidative and inflammatory stress, particularly in obese and diabetic subjects[12,14,25,26]. Postprandial hyperglycerolaemia is associated with enhanced plasma levels of pro-inflammatory cytokines such as TNF-α and IL-6[27–29], and the magnitude of postprandial TAG accumulation is considered to be an independent risk factor for atherosclerosis[30,31]. In the present study, we compared the *in vitro* with the *in vivo* anti-inflammatory properties of BC, chosen due to its high concentration of both polyphenols and ascorbic acid.

In our cultured macrophages, the BC treatment significantly inhibited LPS-induced inflammation indicated by lower mRNA levels of TNF-α, IL-1β and iNOS. Reduced expression of these cytokines may be attributable to the reduced activation of NF-κB since we observed lower nuclear p65 levels in BC-treated cells. Furthermore, CD36 mRNA and intracellular cholesterol were lower in BC-treated cells, suggesting reduced scavenger receptor-mediated cholesterol accumulation in activated macrophages. Taken together, our data indicate that BC potentially attenuates macrophage activation *in vitro*, thereby counteracting chronic inflammatory processes associated with atherosclerosis[32]. Therefore, we developed a smoothie containing BC and other fruits which exhibited antioxidant and anti-inflammatory properties *in vitro* for the human intervention study. In order to meet wide consumer acceptance, a sensory panel evaluated different smoothie recipes in terms of sweetness and bitterness, texture, astringency, etc. The final smoothie recipe contained the highest feasible concentration

of BC purée of 15 %, besides red grape, raspberry and cherry fruit juices and purées. In the present *in vitro* analyses, these fruits were shown to have relatively high amounts of phenolic compounds and ascorbic acid and to exhibit antioxidative or anti-inflammatory properties. Since within the scope of the present study our primary concern was to evaluate potential *in vivo* effects, we focused on the presentation of *in vitro* data on BC.

For the present human pilot intervention study, we recruited men with an atherosclerosis-prone phenotype (including BMI > 25 kg/m^2 and TAG > 1.7 mmol/l) and evaluated the effects of a BC-based beverage (smoothie) on the postprandial progression of blood biomarkers after a high-fat and high-carbohydrate meal. Plasma TAG, glucose and insulin were increased in our subjects after the high-energy meal, while no significant differences were observed between the BC and placebo time curves. Nevertheless, TAG tended to be lower following the BC treatment compared with placebo. This may be of particular importance as the magnitude of postprandial TAG is related to CVD risk[27]. Follow-up studies including higher numbers of subjects are warranted to verify
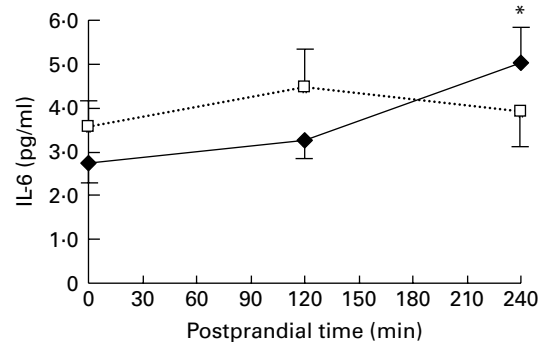
Fig. 5. Circulating IL-6 concentration in the plasma of male volunteers before (0 min) and after (120, 240 min) ingestion of a high-energy meal in combination with a blackcurrant (BC, ◆)-based or placebo (···□···) beverage. Values are means, with standard errors represented by vertical bars (n 10). * Mean values were significantly different ($P<0.05$; paired t tests) for postprandial changes compared with baseline (0 min).

the trend of lower postprandial TAG levels in response to BC consumption observed in the present study.

In our subjects, total cholesterol, HDL- and LDL-cholesterol were significantly decreased in response to the high-fat and carbohydrate meal, an effect that has recently been described in the literature[33,34]. Despite the relatively small number of participants in the present study, we were able to confirm the significant postprandial decline in total cholesterol and LDL-cholesterol of about 0·2–0·3 mmol/l and HDL-cholesterol of about 0·1 mmol/l. There may be two possible explanations for this postprandial decline in cholesterol: increased liver uptake and increased transport of LDL particles from plasma into the arterial wall[33]. The latter would promote athero-sclerotic events and contribute to the proatherogenic pheno-type occurring during the postprandial state. However, no differences in cholesterol levels were observed between the BC and placebo treatments.

Increased low-grade inflammation mainly related to higher circulating levels of pro-inflammatory cytokines is supposed to contribute to the postprandial proatherogenic phenotype. Indeed, meal-induced TAG has been shown to activate leucocytes and inflammatory signalling pathways, such as NF-κB[35,36]. Nevertheless, several lines of evidence also suggest a postprandial decrease in circulating TNF-α levels[37,38]. In the present study, TNF-α and IL-1β were measured in supernatants of *ex vivo* stimulated whole-blood cell cultures and were thus mainly related to leucocyte-mediated inflammation. Both TNF-α and IL-1β were signifi-cantly reduced following the consumption of the placebo test meal. Interestingly, after the BC test meal, TNF-α and IL-1β were also decreased but to a lower extent, which was not significantly different from baseline. This may indicate that the TNF-α-lowering effect elicited by the high-energy meal was attenuated by BC consumption.

In addition, circulating levels of IL-6 were determined in plasma. The role of circulating IL-6 has been controversially discussed with special regard to insulin sensitivity[39,40]. IL-6 may be able to suppress other pro-inflammatory, mainly adipocyte-derived cytokines, induce lipolysis and increase glucose uptake[41,42]. Lack of functional IL-6 is associated with impaired glucose tolerance and hyperglycerolae-mia[39,43]. Therefore, short-term increases in circulating IL-6 levels such as during the postprandial state have been suggested to be beneficial[44]. In the present study, plasma IL-6 was significantly increased after the BC but not after the placebo test meal. However, the role of postprandial circulat-ing pro-inflammatory cytokines is still uncertain and warrants further investigations.

We furthermore examined plasma radical-scavenging capacity (ORAC) and ascorbic acid concentrations in our subjects following test meal consumption. We observed a sig-nificant reduction in both parameters in the placebo group, indicative of an oxidative imbalance in the postprandial state. After BC meal consumption, postprandial ORAC values were likewise decreased (at 30 and 60 min), but increased significantly again after 90 and 120 min. This may be a result of intestinal absorption of ascorbic acid present in high concentrations in the BC beverage. In accordance, plasma ascorbic acid concentrations were significantly higher after BC meal consumption compared with both baseline and placebo meal consumption. Since the increases in plasma ascorbic acid and in ORAC values do not exactly coincide (120 *v.* 90 min), endogenous mechanisms, which modulate the postprandial antioxidant/oxidant balance or dietary factors such as other BC meal-derived compounds, may have contri-buted to increased plasma radical-scavenging capacity. As such, plasma anthocyanin and anthocyanin metabolites were analysed; however, none was detectable. This may be due to the relatively low bioavailability of anthocyanins that has been already described in the literature[15]. In a previous study, the intake of 183 mg anthocyanins resulted in a plasma concentration of only 4 nmol/l[45]. Since the antho-cyanin concentration of the BC juice used in the present study was 11 mg, we assume that the plasma anthocyanin levels did not exceed 1 nmol/l, which was the detection limit of the method used. We did, however, find increased amounts of the predicted gut-derived flavonoid metabolite, 1,3,5-trihydroxybenzene, in plasma 90–120 min postpran-dially, indicating the absorption of possible anthocyanin breakdown products.

Nevertheless, the value of determining radical-scavenging capacity by the ORAC and other related assays may be limited to assess antioxidant power *in vivo*[46]. Furthermore, it has also been partly questioned whether antioxidants actually contribute to the beneficial effects of fruit and vegetables *per se*[47]. Interestingly, in our volunteers, postprandial levels of oxLDL were not different from baseline and also not different between the placebo and BC groups. The activity of paraoxonase, which prevents and reverses LDL oxidation in plasma, was also not different in response to the test meals and between the treatments. As a consequence, we may assume that our subjects were, by and large, not exposed to increased oxidative stress (as indicated by oxLDL) in the postprandial state.

Taken together, BC juice exhibited potent anti-inflammatory properties in cultured macrophages *in vitro*, probably owing to its high polyphenol and ascorbic acid concentrations. In human subjects, consumption of a BC-based juice counter-acted the decrease in plasma radical-scavenging capacity and significantly increased plasma ascorbic acid following a high-fat and -carbohydrate meal. Though effects on the postprandial pro-inflammatory response were not clear, TAG may be attenuated by BC-based juice consumption. Overall, the potential anti-atherogenic effects observed *in vitro* cannot be directly transferred to the postprandial situ-ation *in vivo*, at least partly, due to the very low bioavailability of anthocyanins.

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