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Wild blueberry (*Vaccinium angustifolium*) consumption improves inflammatory status in the obese Zucker rat model of the metabolic syndrome

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Abstract

The metabolic syndrome (MetS) is a major public health problem in the United States. Chronic inflammation is a critical component of the MetS, leading to dramatically increased risk of type II diabetes and cardiovascular disease.

This study investigates the ability of a wild-blueberry-enriched diet to improve the proinflammatory status associated with MetS in the obese Zucker rat (OZR). Circulating levels of key inflammatory markers and their expression in the liver and abdominal adipose tissue were examined in OZR and its genetic control, the lean Zucker rat (LZR), after feeding a control or an 8% wild blueberry diet (WB) for 8 weeks from age 8 to 16 weeks.

In the OZR, WB consumption resulted in decreased plasma concentrations of tumor necrosis factor (TNF)-α (−25.6%, $P<.05$), interleukin (IL)-6 (−14.9%, $P<.05$) and C-reactive protein (CRP) (−13.1%, $P<.05$) and increased adiponectin concentration (+21.8%, $P<.05$). Furthermore, expression of IL-6, TNF-α and nuclear factor (NF)-kB was down-regulated in both the liver (−65%, −59% and −25%, respectively) and the abdominal adipose tissue (−64%, −52% and −65%), while CRP expression was down-regulated only in the liver (−25%). In the abdominal adipose tissue, similar trends were also observed in LZR following WB treatment, with decreased liver expression of NF-kB, CRP, IL-6 and TNF-α (−24%, −16%, −21% and −50%) and increased adiponectin expression (+25%).

Results of this study suggest that wild blueberry consumption exerts an overall anti-inflammatory effect in the OZR, a model of the metabolic syndrome.

Keywords: Metabolic syndrome; Inflammation; Obese Zucker rat; Blueberries

1. Introduction

The metabolic syndrome (MetS) is characterized by the concurrent presence of central obesity, dyslipidemia, insulin resistance, glucose intolerance, hypertension and associated abnormalities such as endothelial dysfunction and pro-oxidative, prothrombotic and proinflammatory status [1,2]. This combination of risk factors dramatically increases the risk of type II diabetes mellitus and coronary heart disease, the latter being the leading cause of death in the United States [2,3].

The obese Zucker rat (OZR) represents a valid experimental model for the human MetS [4]. Due to its genetic profile, it develops between 8 and 20 weeks of age a multitude of detectable abnormalities, including obesity, hypertriglyceridemia and hypercholesterolemia [5], insulin resistance and hyperinsulinemia [6], as well as a moderate form of hypertension [7].

It is now widely recognized that chronic levels of inflammation are implicated in the pathogenesis of a wide variety of chronic conditions including type II diabetes, cardiovascular disease (CVD) and cancer [8]. A proinflammatory state strongly correlates with oxidative stress, endothelial dysfunction, atherosclerosis and insulin resistance, leading to the hypothesis that inflammation could in fact be the underlying factor linking all the different abnormalities of the MetS [8]. High levels of C-reactive protein (CRP) [2], low adiponectin [9] and elevated tumor necrosis factor (TNF)-α and interleukin (IL)-6 [10] contribute to the inflammatory status associated with the MetS. Expression of the above cytokines is regulated by nuclear factor (NF)-kB, an oxidative-stress-sensitive transcription factor which is likely the main link between oxidative stress and chronic inflammation [11].

Diet can potentially modulate inflammatory status by influencing the expression of proinflammatory and anti-inflammatory cytokines and modifying the balance between various eicosanoids with different proinflammatory capacity [8]. Both *in vitro* and in animal models, dietary bioactive compounds such as polyphenols have been shown to affect the expression of genes involved in inflammation and lipid metabolism and modulate the transcriptional activities of different nuclear receptors that control such pathways [12–14].

Wild blueberries are among the commercially available fruits and vegetables that contain the highest levels of polyphenols, mostly anthocyanins (ACNs) [15]. Our past dietary studies have documented their cardioprotective effects on biomechanical and structural characteristics of arteries in both Sprague–Dawley (SD) and spontaneously hypertensive rats [16–18], as well as their antioxidant

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protection against DNA damage in SD lymphocytes [19]. Additional effects of highbush blueberries have been also documented, such as atheroprotection in ApoE-deficient mice [20], lowered blood cholesterol in pigs [21], lowered triglycerides and fasting insulin in OZRs [22], and improvement of insulin sensitivity in obese men and women with insulin resistance [23].

Thus, regular consumption of dietary-achievable amounts of wild blueberries rich in polyphenols may have a significant impact on the inflammatory status associated with the MetS. Hence, the goal of this study is to investigate the ability of a wild-blueberry-enriched diet to improve parameters related to the pathogenesis of the MetS in the OZR.

Specifically, this study examines the effect of wild blueberries on plasma circulating levels and expression in liver and abdominal adipose tissue of representative markers of subclinical inflammatory status: the visceral proteins CRP and adiponectin, the systemic cytokines TNF-α and IL-6, and the transcription factor NF-kB that regulates the expression of all the above cytokines.

2. Materials and methods

2.1. Zucker rats

Twenty male OZR (*fa/fa*) and 20 male lean LZR (*Fa/Fa*) were purchased from Charles River Laboratories (Raleigh, NC, USA). The rats were housed individually in an environmentally controlled room maintained at 22°C, with a 12-h:12-h light:dark cycle, in mesh-bottomed cages to prevent coprophagia.

Ten rats from each strain were randomly assigned to a wild-blueberry-enriched diet (WB), while the other 10 were assigned to a control diet (C) for 8 weeks.

Rats were placed on the diets at 8 weeks of age and subsequently sacrificed at 16 weeks of age. Food intake was recorded daily, and rats were weighed weekly. The experimental protocol was reviewed and approved by the University of Maine Institutional Animal Care and Use Committee.

2.2. Wild blueberries

Wild blueberries were provided as a composite from Wyman's (Cherryfield, ME, USA) and were freeze-dried and powdered with standard procedures (FutureCeuticals, Momence, IL, USA).

The wild blueberry powder was vacuum-packed in plastic bags and stored in the dark at −20°C until use. Analysis demonstrated that the total ACN content of the wild blueberry powder is 1.5% w/w, including 21 different ACNs, mainly malvidin 3-galactoside and peonidin 3-galactoside [24].

2.3. Diets

The control diet was composed of dextrose, egg white solids, vitamin mix, mineral mix, DL-methionine, biotin and corn oil as previously described [16]. For the wild-blueberry-enriched diet, wild blueberry powder was incorporated at 8% w/w to the control diet, substituting for dextrose to maintain the same proportion of all other ingredients. The diets were prepared from the above purified ingredients, stored at 4°C and used within 1 week of preparation.

2.4. Tissue collection

At the end of the experimental period, animals were anesthetized with 95% CO_2/5% O_2 for 2 min. They were quickly exsanguinated by cardiac puncture, and blood was collected for immediate plasma separation, collection and storage at −80°C until subsequent analysis. Liver and abdominal adipose tissues were excised, immediately snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

2.5. Circulating markers of inflammation

Plasma samples were analyzed for markers of inflammation by means of commercially available immunoassay kits.

TNF-α was determined using the Quantikine Rat TNF-α Immunoassay kit (R&D Systems #RTA00), IL-6 was measured using the Quantikine Rat IL-6 Immunoassay kit (R&D Systems #R6000B), adiponectin was measured using the Rat Adiponectin ELISA Kit (Millipore #EZRADP-62K), and CRP was measured using the high-sensitivity Rat CRP ELISA Kit (Millipore #CYT294).

2.6. Expression of CRP, IL-6, TNF-α, adiponectin and NF-kB in liver and adipose tissue

Briefly, mRNA from liver and abdominal adipose tissues was isolated, retro-transcribed to cDNA and subjected to two-step, real-time, reverse transcription polymerase chain reaction (RT-PCR) amplification using rat-specific primer sequences for the CRP, IL-6, TNF-α, adiponectin and NF-kB genes. mRNA from frozen fat fragments was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen #74804), while mRNA from liver was isolated using the RNeasy Mini Kit (Qiagen #74104). Quality and quantity of extracted mRNA were determined spectrophotometrically, measuring absorbance at 260 nm and 280 nm in UV transparent cuvettes. Reverse transcription to cDNA and genomic DNA elimination were performed using the QuantiTect Reverse Transcription Kit (Qiagen #205313).The reverse transcription product was subjected to RT-PCR on a quantitative PCR System (Bio-Rad CFX96) using Sybr Green master mix (SSoFastEvaGreen, Bio Rad #172-5202) and rat-specific primer sequences targeting the genes of interest (Table 1). For each primer and tissue (liver or adipose), the analysis was performed in triplicate with a reaction volume of 20 μl per well (Sybr Green Mix 1×, primers forward and reverse 0.2 μM, reverse transcription product 1.5 μl). After an enzyme activation step (95°C for 30 s), 45 amplification cycles were performed (denaturation at 95°C for 2 s, annealing/extension at 60°C for 5 s) followed by a melting curve (75°C–95°C in 0.5°C increases, 2 s per step) to ensure specificity of amplification. Relative expression of the genes of interest was determined by the ΔΔCt method as described by Livak and Schmittgen [25] relative to a housekeeping gene (beta-actin) and expressed as fold-variation following WB treatment compared to the control animals.

Table 1
List of rat-specific primers used in RT-PCR

Gene	Detected transcript[a]	Details	Amplicon length
Actb	NM_031144	Qiagen #QT00193473	145 bp
Crp	NM_017096	Qiagen #QT00391650	109 bp
Adipoq	NM_144744	Qiagen #QT01169343	84 bp
Tnf	NM_012675	Qiagen #QT00178717	75 bp
Il6	NM_012589	Qiagen #QT00182896	128 bp
Nfkb1	XM_001075876	Qiagen #QT01577975	99 bp

[a] NCBI Reference Sequence.

2.7. Statistical analysis

Results for each of the different parameters under study were evaluated using two-way analysis of variance (ANOVA) with dietary treatment (WB vs. C) and animal model (OZR vs. LZR) as independent factors, and the interaction term diet × model to evaluate the effect of diet within each model. Significant main effects and interactions were further evaluated using Tukey honestly significant difference *post hoc* comparisons. Statistical analysis was performed using R statistical software version 2.15.1 (R Foundation for Statistical Computing, Vienna, Austria). Results were expressed as mean±S.E.M. and considered significant at $P<.05$.

3. Results

Daily food intake was 24.4±2.3 g for LZR and 30.1±2.6 g for OZR. Body weight at the time of sacrifice was 383±20 g for LZR and 583±72 g for OZR, and average weight gain during the 8 weeks of treatment was 168±17 g for LZR and 270±46 g for OZR. No statistically significant difference was observed between the wild blueberry and control groups in the above parameters.

Control OZR at 16 weeks of age had dramatically higher plasma levels of fasting glucose, triglycerides, total cholesterol and non-high-density lipoprotein cholesterol (294.1±14.9, 574.5±31.5, 263.1±12.9 and 121.1±2.4 mg/dl, respectively) compared to control LZR (178.3±17.7, 86.4±11.8, 101.1±8.7 and 49.4±3.7 mg/dl, respectively) [26].

Results for markers of inflammation in plasma are reported in Table 2. IL-6, TNF-α, adiponectin and CRP were all significantly higher

Table 2
Fasting plasma levels of inflammatory markers in lean and obese Zucker rats following control or a wild blueberry diet[a]

	LZR-C	LZR-WB	OZR-C	OZR-WB
IL-6 (pg/ml)	189.4±7.9	176.6±6.7	257.7±7.7 $^{+}$	219.3±8.0**
TNF-α (pg/ml)	8.3±0.4	7.3±0.5	38.7±0.9 $^{+}$	28.8±0.8***
Adiponectin (μg/ml)	12.1±0.9	12.6±0.9	17.0±1.1 $^{+}$	20.7±1.5 *
CRP (μg/ml)	242.0±11.2	226.0±8.6	438.5±13.9 $^{+}$	381.0±11.1*

[a] Values are means±S.E.M., $n=10$. Asterisks indicate statistically significant differences of OZR-C vs. OZR-WB ($^{*}P<.05$; $^{**}P<.01$; $^{***}P<.001$). "+" indicates statistically significant difference LZR-C vs. OZR-C ($P<.001$).

in the OZR group compared to the LZR, independent of diet. Wild blueberry consumption in the OZR group resulted in significantly lower levels of IL-6 and TNF-α compared to the control (−14.9% and −25.6%, respectively), whereas adiponectin levels significantly increased (+21.8%). Similar trends were observed in the LZR group, although a two-way ANOVA did not reveal statistically significant differences with wild blueberry consumption.

Results for the genetic expression of molecules related to inflammatory status are reported in Fig. 1. In the liver, expression of IL-6, TNF-α and NF-kB was significantly lower in the wild blueberry group compared to the control in OZR (−65%, −59% and −25%, respectively). Similar trends for these markers were observed in the LZR liver following the WB diet, although only NF-kB expression reduction was statistically significant (−24%). In the abdominal adipose tissue, expression of IL-6, TNF-α and NF-kB was significantly lower in the wild blueberry group compared to the control in the OZR (−64%, −52% and −65%, respectively), but not in the LZR. Furthermore, expression of IL-6, TNF-α and NF-kB in both the liver and the adipose tissue was markedly increased in the OZR on the control diet compared to the LZR, and this effect was almost completely reversed by the wild blueberry treatment. Liver expression of CRP was, on average, lower in the OZR compared to the LZR, independent of dietary treatment. However, the wild blueberry diet significantly decreased CRP expression in both groups (−16% in LZR and −25% in OZR). Expression of adiponectin in the abdominal adipose tissue was significantly higher in the OZR group compared to the LZR, independent of diet. Furthermore, wild blueberry consumption resulted in significantly increased adiponectin expression in the LZR (+25%), but not in the OZR. Expression of adiponectin in hepatocytes and CRP in adipocytes was too low to generate usable PCR data and was therefore not considered in the analysis.

4. Discussion

This study documents for the first time that 8 weeks of dietary treatment with wild blueberries significantly and positively impacts plasma levels and expression of representative markers of inflammation, resulting in an overall attenuation of the inflammatory status in the OZR.

The metabolic abnormalities of the OZR, similar to those observed in the human MetS, are accompanied by a profound prooxidant, prothrombotic and proinflammatory state, resulting in higher circulating levels of proinflammatory cytokines [27]. Indeed, in the present study, circulating levels of CRP, IL-6 and TNF-α were all significantly higher, whereas adiponectin was lower, in the OZR group compared to their littermate controls (the LZR group), independent of diet.

CRP is a useful marker of subclinical chronic inflammation and a predictor of CVD [28], insulin resistance, diabetes mellitus as well as

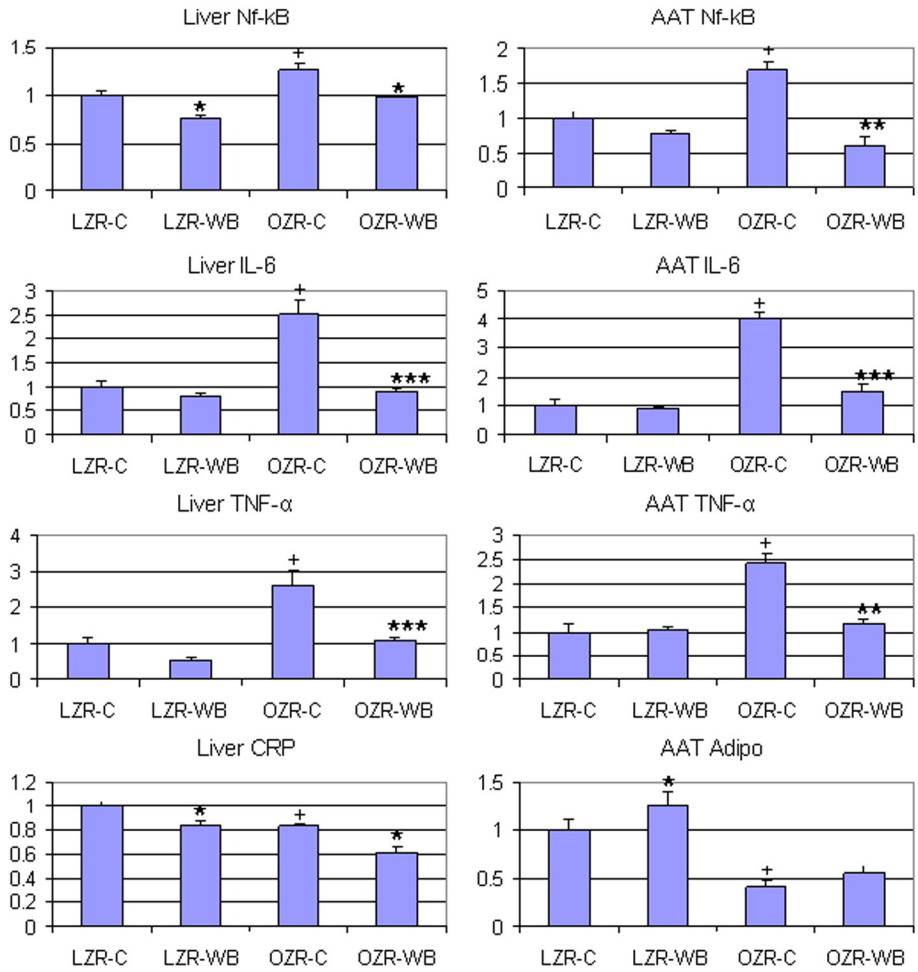

Fig. 1. Relative expression of genes related to the inflammatory status in lean and obese Zucker rats following control or a wild blueberry diet. Values are means±S.E.M., expressed as $2^{-\Delta\Delta Ct}$ normalized to beta-actin and relative to LZR-C. n=10. Asterisks indicate statistically significant effects of diet within the same model, (*P<.05; **P<.01; ***P<.001). "+" indicates statistically significant difference of LZR-C vs. OZR-C (P<.001). Abbreviation: AAT, abdominal adipose tissue.

MetS [29]. Interestingly, in our study, higher CRP levels were found in plasma, while liver expression of CRP was on average lower in the OZR compared to the LZR, independent of diet. Furthermore, wild blueberry consumption significantly decreased CRP plasma levels in the OZR and CRP liver expression in both groups. Indeed, plasma CRP concentrations appear to be inversely associated with ACN intake among adults in the United States when National Health and Nutrition Examination Survey food consumption data are analyzed for flavonoid content [30], and decreased levels of serum CRP were measured in hypercholesterolemic individuals after 24 weeks of consumption of a purified ACN mixture (320 mg twice a day) [31].

Recent human intervention studies investigating the effects of highbush blueberry consumption [32,23] did not detect significant variations in circulating levels of CRP since the inflammatory status of these individuals was already low at baseline. To our knowledge, the effect of polyphenol-rich berry consumption has not been evaluated to date in humans with high levels of subclinical inflammation, whereas the present study utilized an animal model of MetS and inflammation.

Beyond their local activity, TNF-α and IL-6 have major systemic effects when produced either acutely in large amounts or chronically in lesser amounts, as in the case of MetS [33].

Chronically elevated IL-6 levels are common in patients with cardiovascular pathologies [34], contributing to cell damage, oxidative stress, blood clotting and atherothrombotic events [35]. Elevated TNF-α levels are associated with endothelial dysfunction, atherosclerosis and obesity, with the vasculature and adipose tissue being major sites of its production [36].

Results from our gene expression experiments for the above markers are, in general, consistent with the circulating levels that we found in plasma. The wild-blueberry-enriched diet significantly reversed the increased plasma levels of IL-6 and TNF-α observed in OZR by down-regulating the expression of these proinflammatory cytokines in both the liver and the abdominal adipose tissue. Decreased TNF-α expression was previously reported in mice fed a high-fat diet and supplemented with ACN-rich extracts [37,38]. Addition of 4% whole blueberry powder for 8 weeks to a high-fat diet of mice also resulted in reduced expression of TNF-α in adipocytes, although IL-6 expression did not change [39]. Interestingly, OZR fed either a low- or a high-fat diet containing 2% highbush blueberry powder for 90 days did not have any significant variation in plasma IL-6 and TNF-α [22], suggesting that the dose may have been too low to produce an observable effect on these markers. Furthermore, it has been well established that wild blueberries are consistently higher in ACN, total phenolics and antioxidant capacity compared with highbush blueberries [40].

In our study, wild blueberry consumption increased adiponectin circulating levels in the OZR group, although no significant difference was observed in adiponectin mRNA levels in the abdominal adipose tissue. A moderate increase in adiponectin expression was observed in LZR instead.

Low plasma adiponectin levels are significantly correlated with endothelial dysfunction and considered an independent risk factor for type II diabetes and coronary heart disease [41]. Proinflammatory cytokines such as IL-6 and TNF-α inhibit adiponectin production in the adipocytes [42], whereas adiponectin decreases cytokine production from macrophages by inhibiting NF-kB signaling through cAMP-dependent pathways [43].

A negative association between abdominal adiposity and mRNA levels of adiponectin has been consistently reported in human subjects [44].

Similarly to humans, in this study, mRNA adiponectin in the abdominal adipose tissue was significantly lower in the OZR group compared to the LZRs. On the other hand, circulating adiponectin levels were higher in our OZRs compared to the LZRs. The discrepancy between the circulating and the adipose levels could be explained, at least partially, by considering that OZRs have several-fold more adipose tissue than LZRs. Therefore, the total amount of circulating adiponectin may still be higher in the OZRs, although the amount produced per unit of fat tissue may be lower.

Among the mechanisms proposed to explain the anti-inflammatory actions of ACNs and polyphenols, inhibition of NF-kB activation is a potential candidate [45,46]. ACN supplementation was documented to inhibit NF-kB and suppress inflammatory markers in human monocytes as well as healthy adults [47]. Although NF-kB activity was not determined in this study, mRNA expression of NF-kB itself was significantly higher in OZR compared to LZR, independent of treatment, which could explain the overall proinflammatory environment observed in these animals. Furthermore, wild blueberry treatment resulted in decreased NF-kB expression in both the liver and the abdominal adipose tissue of OZR. Seymour et al. [48] have reported that adipose tissue NF-kB activity was also decreased in OZR fed a high-fat diet and supplemented with 1% freeze-dried whole tart cherry powder for 90 days, along with decreased IL-6 and TNF-α mRNA.

Activation of functional transcription activity of NF-kB induced by oxidative stress is a key step leading to up-regulation of proinflammatory molecule expression, such as TNF-α and IL-6, and possibly down-regulation of anti-inflammatory molecules, such as adiponectin [49]. Hence, it can be hypothesized that the anti-inflammatory effect observed for antioxidant molecules, such as polyphenols, may be dependent on inhibition of NF-kB activation, leading to a reduction of proinflammatory cytokines and increase of anti-inflammatory mediators such as adiponectin. Attenuation of NF-kB activation could be related to the antioxidant capacity of blueberries, thereby providing a potential association with the observed anti-inflammatory effect of wild blueberry intake.

The present study examined for the first time the effects of medium-term, dietary-achievable wild blueberry consumption in an animal model of the human MetS. A clear reduction in circulating levels of markers of inflammatory status was observed, as well as their reduced expression levels, in both the adipose tissue and the liver. For most of the markers under investigation, improvements were also observed in the littermate controls, in particular with regard to liver expression of inflammatory molecules.

Thus, the documented anti-inflammatory effect of wild blueberry diets on the OZR model may have implications for the human MetS, suggesting a nonpharmacologic approach in preventing and/or improving risk factors of the MetS and its associated cardiometabolic abnormalities.

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