Specific Dietary Polyphenols Attenuate Atherosclerosis in Apolipoprotein E–Knockout Mice by Alleviating Inflammation and Endothelial Dysfunction

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Objective—Animal and clinical studies have suggested that polyphenols in fruits, red wine, and tea may delay the development of atherosclerosis through their antioxidant and anti-inflammatory properties. We investigated whether individual dietary polyphenols representing different polyphenolic classes, namely quercetin (flavonol), (−)-epicatechin (flavan-3-ol), theaflavin (dimeric catechin), sesamin (lignan), or chlorogenic acid (phenolic acid), reduce atherosclerotic lesion formation in the apolipoprotein E (ApoE)−/− gene–knockout mouse.

Methods and Results—Quercetin and theaflavin (64-mg/kg body mass daily) significantly attenuated atherosclerotic lesion size in the aortic sinus and thoracic aorta (P<0.05 versus ApoE−/− control mice). Quercetin significantly reduced aortic F2-isoprostane, vascular superoxide, vascular leukotriene B4, and plasma-sP-selectin concentrations; and augmented vascular endothelial NO synthase activity, heme oxygenase-1 protein, and urinary nitrate excretion (P<0.05 versus control ApoE−/− mice). Theaflavin showed similar, although less extensive, significant effects. Although (−)-epicatechin significantly reduced F2-isoprostane, superoxide, and endothelin-1 production (P<0.05 versus control ApoE−/− mice), it had no significant effect on lesion size. Sesamin and chlorogenic acid treatments exerted no significant effects. Quercetin, but not (−)-epicatechin, significantly increased the expression of heme oxygenase-1 protein in lesions versus ApoE−/− controls.

Conclusions—Specific dietary polyphenols, in particular quercetin and theaflavin, may attenuate atherosclerosis in ApoE−/− gene–knockout mice by alleviating inflammation, improving NO bioavailability, and inducing heme oxygenase-1. These data suggest that the cardiovascular protection associated with diets rich in fruits, vegetables, and some beverages may in part be the result of flavonoids, such as quercetin. (Arterioscler Thromb Vasc Biol. 2010;30: 00-00.)

Key Words: polyphenol ■ NO ■ soluble P-selectin ■ endothelin-1 ■ oxidative stress ■ inflammation ■ endothelial dysfunction

Atherosclerosis is a multifactorial disease developing over many years, with symptoms becoming apparent in the late stages of the disease. Inflammation, oxidative stress, and endothelial dysfunction are associated with the pathogenesis of atherosclerosis. Polyphenols are naturally occurring components of fruits and vegetables and are currently the focus of much nutritional and therapeutic interest. The results of population studies suggest that adopting polyphenol-rich diets may protect against cardiovascular disease, whereas animal and human intervention studies indicate cardiovascular protective effects of polyphenol-rich foods. Mechanisms by which these compounds exert their cardiovascular protective effects are inconclusive. It is widely hypothesized that dietary polyphenols protect against atherosclerosis by preventing 1 or more of the processes involved in disease progression, such as oxidative stress, inflammation, and endothelial dysfunction. However, there are many hundreds of polyphenols in the human diet, and it is not yet known if some compounds offer more cardiovascular protection than others.

We selected several common dietary polyphenols (structures in Figure 1), such as quercetin (a flavonol found in the diet from fruits, vegetables, and tea), (−)-epicatechin (a flavan-3-ol from cocoa and tea), theaflavin (a dimeric catechin from black tea), sesamin (a lignan from sesame seeds), and chlorogenic acid (a phenolic acid from coffee and some fruits) for this study. Grape polyphenols (including quercetin) have been shown to improve the lipoprotein profile and
reduce plasma inflammatory biomarkers and oxidized low-density lipoprotein (LDL) in healthy human subjects, which may decrease cardiovascular disease risk. Quercetin reduces blood pressure and improves endothelial function in the rat. Previous human intervention studies have indicated that (−)-epicatechin from cocoa improves endothelial function and reduces inflammation. In a short-term human intervention study, we demonstrated that quercetin and, to a lesser extent, (−)-epicatechin are able to augment NO production and reduce endothelin-1 (ET-1), whereas epigallocatechin gallate had no effect. In vitro studies with leukocytes indicate that the anti-inflammatory activity of flavonoids may be dissociated from their antioxidant activity. Theaflavin was included in our study because it is a major constituent of black tea, which is widely consumed in Western countries and may offer similar antioxidant potency as green tea catechins. Black tea consumption has been shown to improve endothelial function in patients with coronary artery disease. Sesamin is a bioactive lignan in sesame seeds; it may decrease cardiovascular disease risk. Quercetin reduces plasma triglycerides and reduces inflammation. In a short-term human intervention study, we demonstrated that quercetin and, to a lesser extent, (−)-epicatechin are able to augment NO production and reduce endothelin-1 (ET-1), whereas epigallocatechin gallate had no effect. In vitro studies with leukocytes indicate that the anti-inflammatory activity of flavonoids may be dissociated from their antioxidant activity. Theaflavin was included in our study because it is a major constituent of black tea, which is widely consumed in Western countries and may offer similar antioxidant potency as green tea catechins. Black tea consumption has been shown to improve endothelial function in patients with coronary artery disease. Sesamin is a bioactive lignan in sesame seeds; it may decrease cardiovascular disease risk. Quercetin reduces plasma triglycerides and reduces inflammation.

**Methods**

**Materials**

Chemicals and reagents were purchased from Sigma Aldrich, St Louis, Mo; and Cayman Chemical, Ann Arbor, Mich. High-purity solvents were from Univar (Western Australia). Sesamin and theaflavin were provided by Suntory (Japan) and Unilever (Netherlands), respectively. All compounds had greater than 95% purity based on high-performance liquid chromatographic analysis.

**C57BL/6J and ApoE−/− Mice**

The present study was approved by and performed under the guidelines of the Animal Ethics Committees of the University of Western Australia and Royal Perth Hospital. A total of 150 four-week-old male ApoE−/− mice and 25 C57BL/6J mice were obtained from the Animal Resource Centre, Canningvale, Australia. The genetic background for the ApoE−/− is C57BL/6J. They have been backcrossed to the C57BL/6J 10 times. The mice were housed in groups of 5 and placed on a nonpurified stock diet of AIN, 93 mol/L (Glenforest Stockfeeds, Western Australia) (calculated nutritional parameters in Supplemental Table 1; available online at http://atvb.ahajournals.org). A total of 125 ApoE−/− mice were randomized to receive quercetin, (−)-epicatechin, theaflavin, sesamin, or chlorogenic acid (1.3 mg/d; 64-mg/kg body mass; n=25 for each treatment group). These levels are approximately equivalent to 350 mg/d in humans. The treatment compounds were blended with the mouse feed, which was ground into pellets and stored at 0°C until used. The control groups of 25 ApoE−/− mice and 25 C57BL/6J wild-type mice received the blended and pelleted mouse feed with no compounds added. The mice began to receive the prescribed treatment at the age of 6 weeks, until the end of the study. Food, fluid intake, and body weight were monitored on a regular basis throughout the study. Urine was collected from each group in metabolic cages at the ages of 16 and 26 weeks. After 10 weeks of treatment, when the mice were aged 16 weeks, 5 from each group were killed for analysis of early lesion development and plasma and aortic biochemistry studies. The remaining mice were killed for the same analyses at the age of 26 weeks (ie, after 20 weeks of treatment). Animal numbers were based on the power analysis performed on the desired end points (P<0.008 for multiple comparisons between the treatment and control groups) and on a previous study that showed significant differences in lesion area by the age of 26 weeks.

**Plasma and Aortic Tissue Sampling**

Nonfasting mice from each group were studied at the ages of 16 weeks (n=5) and 26 weeks (n=20). Mice were anesthetized with pentobarbital sodium (Nembutal), and the abdominal and thoracic cavities were opened by ventral incision. A blood sample was obtained via vena cava puncture and collected into 50-μL EDTA, 1 g/10 mL in 0.9% saline. The blood plasma was stored after the addition of butylated hydroxytoluene, 8 μg/mL, at −80°C. The aortie sinus and the thoracic and abdominal aorta were removed and stripped of any external fatty deposits. Aortas for histopathologic and biochemical analysis were prepared as previously described.

**Histological Analysis of Mouse Aorta Specimens**

The size of atherosclerotic lesions in the mouse aorta was determined by measuring the cross-sectional lesion area using procedures described previously. The aorta was rinsed in phosphate-buffered saline (PBS) after removal from the phosphate-buffered formaldehyde (4% by volume; pH, 7.0 to 7.3) and processed as described in the Supplemental Material. For immunohistochemistry of heme oxygenase-1 (HO-1), paraffin-embedded thoracic aorta specimens were sectioned every 400 μm, proximally from the third pair of intercostal arteries, for a total distance of 2800 μm. HO-1 protein was detected with anti–rat HO-1 polyclonal rabbit antibody (SPA-895; Stressgen, Ann Arbor, Mich) (final concentration, 10 μg/mL), applied for 24 hours at 4°C. Bound HO-1 antibody was detected with biotinylated anti–rabbit goat IgG (Dako, Glostrup, Denmark) (final concentration, 3.5 μg/mL), applied for 1 hour at room temperature, and the ABC detection method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, Calif). Counterstaining was achieved with hematoxylin and Scott blue solution.
Plasma Cholesterol and Aortic Fatty Acid Composition

The total cholesterol content of the mouse plasma samples was measured using a commercially available cholesterol assay kit (Boehringer Mannheim). Aortic tissue was thawed, weighed, and homogenized in 2 mL of PBS and extracted using ice-cold Folch solution (chloroform-methanol, 2:1 by volume containing 0.1-mmol/L butylated hydroxytoluene). The chloroform layer (containing the F₂-isoprostanes and fatty acids) was collected, dried, and analyzed, as described in detail in the Supplemental Material. For the distribution of lipoprotein classes, mouse plasma was fractionated by fast protein liquid chromatography using 2 columns (Superose HR6 10/30; Amersham Biosciences, Uppsala, Sweden) in series at a flow rate of 30 mL/h. Plasma samples from 5 mice in the same group (80 µL each) were pooled to give 400 µL, were filtered with 0.45 µm filter; 450 µL of diluted plasma was applied to the column and eluted with PBS containing 1-mmol/L EDTA (pH, 7.2). Fractions, 0.5 mL, were collected into tubes containing butylated hydroxytoluene, 20 µL, at 4 mg/mL. Protein was monitored continuously by absorbance at 280 nm, and total cholesterol was measured in each of the fractions using an enzymatic method (Roche Diagnostics GmbH, Germany).

Oxidative Stress

Vascular oxidative stress was assessed by measuring F₂-isoprostanes in aortic tissue by gas chromatography-mass spectrometry using a previously described method.²³ Aortic F₂-isoprostanes were measured in lipid extracts from the mouse aortas and corrected for arachidonic acid. Vascular superoxide was assessed by lucigenin-derivated chemiluminescence,²⁴ as described in detail in the Supplemental Material. The chemiluminescence signal was inhibited by 90% in control incubations, with superoxide dismutase added.

Ex Vivo Vascular Leukotriene B₄ Production

The effect of the polyphenol treatments on 5-lipoxygenase enzyme activity was determined by measuring vascular leukotriene B₄ (LTB₄) production ex vivo, as described in detail in the Supplemental Material.

Plasma-Soluble P-Selectin

The long-term effects of treatment on platelet reactivity were investigated by measuring the plasma concentrations of soluble P-selectin (sP-selectin) using a commercially available mouse sP-selectin enzyme immunoassay kit (R&D Systems, Mnns).

Vascular Endothelial NO Synthase Activity, Urinary Nitrite, Nitrate, and ET-1

NOS activity in aortic homogenates was determined by monitoring the conversion of L-¹[H]arginine to L-¹[H]citrulline using an NOS activity assay kit (Cayman Chemical). Results were expressed as picomoles of L-citrulline per milligram of protein per 60 minutes. Nitrite and nitrate concentrations in urine were determined using a previously published gas chromatographic-mass spectrometric method.²⁵ Results were corrected for creatinine levels. ET-1 was measured in urine with a commercially available ET-1 (mouse) enzyme immunoassay kit (Assay Design, Ga.). ET-1 concentrations were corrected for creatinine levels.

Statistical Analysis

Statistical analyses were performed using SPSS version 15 (SPSS Inc, Chicago, Ill). Data are presented as mean±SEM. A 1-way ANOVA²⁶ with post hoc analyses using the Tukey honestly significant difference test was used to compare treatments. Initially, the ApoE⁻/⁻ control mice were compared with the C57BL/6J mice. The effect of polyphenol treatments in the ApoE⁻/⁻ mice were then compared with effects in the ApoE⁻/⁻ control mice. The results analyzed were considered significantly different when P<0.05.

Results

Animals and Polyphenol Diets

There was no difference in body mass between C57BL/6J and ApoE⁻/⁻ mice over 26 weeks (from a mean body mass of 9.5±0.5 g at week 4 to 28.0±0.3 g at week 26). The daily intake of the polyphenols was calculated for each group based on the daily consumption of the mouse feed (mean daily intake in mg/kg of body mass: quercetin, 63.8±0.5; (−)-epicatechin, 64.0±0.3; theaflavin, 63.5±0.8; sesamin, 63.7±0.4; and chlorogenic acid, 63.8±0.6). No significant difference between the groups was observed.

Aortic Lesion Analyses

The lesion area in the transverse section of the aorta was expressed as a percentage of the lesion to the total area of the aortic tissue. No significant atherosclerotic lesion was observed in C57BL/6J and ApoE⁻/⁻ mice at the age of 16 weeks (data not shown). At the age of 26 weeks, ApoE⁻/⁻ mice exhibited significantly greater lesions at the aortic sinus and the thoracic aortic region just below the aortic arch compared with the C57BL/6J control mice (Figure 2A and B; P<0.05 versus C57BL/6J mice). Lesion formation at both locations was significantly reduced in ApoE⁻/⁻ mice fed a diet containing quercetin or theaflavin (Figure 2A and B, 60% to 80% and 45% to 55%, respectively; P<0.05 versus ApoE⁻/⁻ control mice). Examples of micrographs for aortic cross sections stained for lipid lesions are shown in Supplemental Figure I (available online at http://atvb.ahajournals.org). Treatment with (−)-epicatechin, sesamin, and chlorogenic acid appeared to diminish lesion formation (14%, 40%, and 29%, respectively, versus ApoE⁻/⁻ mice); however, these differences were not statistically significant.

In a subgroup of mice, the expression of HO-1 protein in aortic sections was examined. Aortic sections from apoE⁻/⁻ mice showed greater staining for HO-1 than those of C57BL/6J mice, with HO-1 staining limited largely to atherosclerotic lesions. In apoE⁻/⁻ mice fed the quercetin-supplemented diet, HO-1 protein was significantly increased compared with either the apoE⁻/⁻ control (P<0.05) or the epicatechin-fed apoE⁻/⁻ mice (P<0.01) (Figure 3).

Plasma Cholesterol and Aortic Fatty Acid Composition

ApoE⁻/⁻ mice had significantly elevated plasma concentrations of cholesterol compared with the C57BL/6J mice (Supplemental Table II). The polyphenols did not significantly affect plasma cholesterol concentrations or lipoprotein distribution (Supplemental Figure II) in the ApoE⁻/⁻ mice after 20 weeks of treatment. None of the polyphenol treatment exerted a significant effect on the fatty acid composition in the aorta compared with the control apoE⁻/⁻ mice (data not shown).

Vascular Oxidative Stress

At the age of 26 weeks, ApoE⁻/⁻ mice had significantly higher concentrations of aortic F₂-isoprostanes than C57BL/6J mice (Figure 4A, P<0.05). Diets incorporating quercetin or (−)-epicatechin significantly reduced aortic F₂-isoprostane concentrations in ApoE⁻/⁻ mice (Figure 4A,
Theaflavin, sesamin, and chlorogenic acid treatments did not show any significant effect on aortic F2-isoprostane concentrations. Aortic tissues from the ApoE−/− mice had a slightly elevated superoxide level compared with C57BL/6J mice at the age of 26 weeks (Figure 4B), although this was not significantly different. Quercetin and (−)-epicatechin treatments significantly attenuated vascular superoxide (Figure 4B, P<0.05 versus ApoE−/− control mice); no significant effect was observed for the other treatments.

Ex Vivo Vascular LTB4 Production and Plasma-sP-Selectin
Aortic tissues from ApoE−/− mice produced significantly higher amounts of LTB4 compared with the C57BL/6J mice (Figure 4C, P<0.05). Quercetin and theaflavin treatment significantly reduced LTB4 in the aortic tissues (Figure 4C, P<0.05).

ApoE−/− mice expressed significantly higher plasma concentrations of sP-selectin than C57BL/6J mice at the age of 26 weeks (P<0.005) (Figure 4D). Treatment with quercetin, (−)-epicatechin, and theaflavin significantly lowered the plasma sP-selectin concentrations (P<0.005 versus ApoE−/− control mice). Sesamin and chlorogenic acid did not significantly affect plasma sP-selectin concentrations compared with the ApoE−/− control mice (Figure 4D).
Vascular Endothelial NO Synthase Activity and Urinary Nitrite, Nitrate, and ET-1

At the age of 26 weeks, the vascular endothelial NO synthase (eNOS) activity of ApoE⁻/⁻ mice in the control group was significantly lower compared with the C57BL/6J mice (P<0.05, Figure 5A). Quercetin and theaflavin significantly increased eNOS activity in the aortic tissues (P<0.05 versus ApoE⁻/⁻ control mice, Figure 5A), whereas the other polyphenols showed insignificant elevations in eNOS activity. The increase in eNOS activity significantly correlated with the elevation in excretion of nitrate in the polyphenol-treated ApoE⁻/⁻ mice at 26 weeks (R=0.65, P<0.001). All 5 treatments elevated the urinary nitrate concentration, with quercetin- or theaflavin-treated ApoE⁻/⁻ mice having significantly higher urinary nitrate concentrations compared with the ApoE⁻/⁻ control mice (P<0.05, Supplemental Table III). However, there was no significant difference in urinary nitrite between any of the treatment groups (Supplemental Table III).

Urinary concentrations of ET-1 were significantly increased in the ApoE⁻/⁻ mice at the age of 26 weeks compared with C57BL/6J mice (P<0.05); this was attenuated with quercetin and (−)-epicatechin dietary treatments (P<0.05, Figure 5B).

Correlation Between Lesion Size and Biomarkers

Lesion size at the aortic sinus was significantly correlated with aortic F₂-isoprostanes (R=0.29, P<0.001), aortic eNOS activity (R=−0.67, P<0.001), and urinary nitrate (R=−0.52, P<0.005); the lesion amounts at the thoracic aorta were significantly correlated to aortic F₂-isoprostanes (R=0.22, P<0.01), aortic superoxide (R=0.20, P<0.05), aortic LTB₄ (R=0.21, P<0.01), aortic eNOS activity (R=−0.38, P<0.05), and urinary nitrate (R=−0.38, P<0.05) (Supplemental Table IV).

Discussion

Our study has shown that particular dietary polyphenols are bioactive molecules that can inhibit the development of atherosclerosis. In particular, quercetin and theaflavin significantly attenuated lesion formation in ApoE⁻/⁻ mice. Previous studies have shown that polyphenol-rich beverages, such as red wine, dealcoholized red wine, and tea, can inhibit atherosclerosis in ApoE⁻/⁻ mice. These beverages contain a complex mixture of polyphenolic compounds. Our study suggests that certain individual polyphenols can attenuate atherosclerosis, and these compounds may represent some of the active components of polyphenol-rich beverages. Compounds such as quercetin and theaflavin, which inhibit atherosclerosis, may do so through many pathways, including inhibition of inflammation (LTB₄), improvement of NO bioavailability, and propagation of HO-1 expression (Table).

Lipid peroxidative damage may be a critical step in the pathogenesis of atherosclerosis. The well-recognized antioxidant activity of many polyphenols has led to the proposal that polyphenol protection against atherosclerosis may involve their antioxidant properties. Quercetin and catechins in red wine and tea have been shown to inhibit atherosclerosis.
Black tea consumption decreased lipoprotein oxidation and oxidative stress in apoE<sup>−/−</sup> mice, and in other experimental models.

Hypercholesterolemia is well established as a risk factor for atherosclerosis, and it is possible that dietary polyphenols may protect against the disease by exerting hypocholesterolemic effects. However, animal and clinical studies<sup>21,26</sup> are not conclusive. The polyphenols used in our study had no significant effect on plasma total cholesterol concentrations or lipoprotein distribution. This result is consistent with previous studies<sup>21,22,28</sup> in the apoE<sup>−/−</sup> mouse. The absence of hypocholesterolemic activity suggested that the observed antatherogenic effects of quercetin and theaflavin were independent of serum cholesterol in this study.

Leukocyte adhesion to endothelial cells and their subsequent infiltration into subendothelial spaces are mediated by various adhesion molecules, such as P-selectin and vascular cell adhesion molecule-1, which are expressed on leukocytes, platelets, and endothelial cells. Plasma sP-selectin levels associated with preclinical atherosclerosis in hypercholesterolemic men, and elevated plasma levels of sP-selectin were reported in high-risk patients with hypercholesterolemia<sup>34</sup> and hypertension. ApoE<sup>−/−</sup> mice have significantly higher plasma concentrations of sP-selectin than C57BL/6J control mice. In our study, dietary treatment with quercetin and theaflavin significantly lowered plasma sP-selectin yet did not significantly decrease atherosclerosis at either the aortic root or the thoracic aorta, indicating that inhibition of P-selectin expression may not be sufficient for polyphenols to inhibit atherosclerosis.

Inflammation is recognized as a key process in atherosclerosis.<sup>1,2</sup> Inflammatory processes in the vascular wall may be mediated by a range of factors, such as cytokines, eicosanoids, and NO, which in turn modulate cellular signaling, cell growth, differentiation, and a variety of other cellular processes. There is evidence that LTB4, a potent chemotactic molecule, is involved in arterial leukocyte recruitment.<sup>41</sup> LTB4 signaling through nuclear factor-κB–dependent BLT1 receptors on vascular smooth muscle promotes atherosclerosis and intimal hyperplasia,<sup>42</sup> whereas knockdown of the BLT1 receptor has been shown to reduce lesion formation in achievable human intake. Oxidative stress in the vasculature was effectively attenuated by quercetin, as demonstrated by significant reduction of aortic F2-isoprostanes and superoxide (Figure 4). Similarly, (−)-epicatechin also significantly decreased aortic F2-isoprostanes and superoxide. However, in the case of this polyphenol, inhibition of oxidative stress in the vessel was not associated with a significant reduction in aortic lesion size. Furthermore, theaflavin significantly attenuated atherosclerosis but did not significantly reduce vascular F2-isoprostanes and superoxide. Therefore, the inhibition of oxidative stress is not generally sufficient, nor is it a necessity, for polyphenols to inhibit atherosclerosis in ApoE<sup>−/−</sup> mice. This interpretation is consistent with the overall weak correlations between lesion size and markers of oxidative stress in the present study, in previous reports<sup>2,21,22,34,35</sup> of a dissociation of atherosclerosis and arterial lipoprotein lipid oxidation and oxidative stress in apoE<sup>−/−</sup> mice, and in other experimental models.

In ApoE<sup>−/−</sup> mice while also reducing LDL susceptibility to oxidation.<sup>27,29</sup> Quercetin-3-O-glucuronide (a major in vivo quercetin metabolite) was shown to localize within activated macrophages in human atherosclerotic lesions and to prevent the uptake of oxidized LDL through the downregulation of scavenger receptors.<sup>30</sup> Black tea consumption decreased lipoprotein oxidation in New Zealand white rabbits and theaflavin, a major polyphenol present in black tea, was shown to be as effective as catechins as in vitro antioxidants.<sup>16</sup> The ingestion of sesame (in which sesamin is a major lignan) affected antioxidant status and reduced lipids in postmenopausal women.<sup>17</sup> Chlorogenic acid and its major in vivo metabolite, caffeic acid, have antioxidant effects in vitro,<sup>32</sup> whereas the consumption of coffee (a major dietary source of chlorogenic acid) resulted in the incorporation of phenolic acids into LDL and increased the resistance of LDL to ex vivo oxidation.<sup>33</sup> The polyphenols tested in our study were incorporated into the diet and given at a dose corresponding to

**Figure 5.** A, Vascular eNOS activity of C57BL and ApoE<sup>−/−</sup> mice after 20 weeks (mice aged 26 weeks) of different dietary treatments: C57BL/control (n = 5), ApoE/control (n = 5), ApoE/quercetin (n = 5), ApoE/(−)-epicatechin (n = 5), ApoE/theaflavin (n = 5), ApoE/sesamin (n = 5), and ApoE/chlorogenic acid (n = 5). B, Urinary ET-1 concentrations of C57BL and ApoE<sup>−/−</sup> mice after 20 weeks (mice aged 26 weeks) of different dietary treatments: C57BL/control (n = 5), ApoE/control (n = 5), ApoE/quer- cetin (n = 5), ApoE/(−)-epicatechin (n = 5), ApoE/theaflavin (n = 5), ApoE/sesamin (n = 5), and ApoE/chlorogenic acid (n = 5). In A and B, bars with the same subscript are not significantly different from each other using 1-way ANOVA analysis with the Tukey honestly significant difference post hoc analysis.
Table. Effects of Specific Polyphenols on Tested Pathways at Week 26*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Quercetin</th>
<th>Epicatechin</th>
<th>Theaflavin</th>
<th>Sesamin</th>
<th>Chlorogenic Acid</th>
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<tr>
<td>Aortic sinus lesion formation</td>
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<td>−14</td>
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<td>−56†</td>
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<td>−43</td>
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<td>−2</td>
<td>−19</td>
<td>9</td>
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<td>Aortic F2-isoprostanes</td>
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<td>−77†</td>
<td>−39</td>
<td>−27</td>
<td>9</td>
</tr>
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<td>HO-1 protein</td>
<td>190†</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Aortic superoxide</td>
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<td>−41†</td>
<td>−10</td>
<td>−24</td>
<td>−15</td>
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<tr>
<td>Aortic LTB4</td>
<td>−53†</td>
<td>−34</td>
<td>−47†</td>
<td>−18</td>
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<tr>
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<td>−33†</td>
<td>−26†</td>
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<td>−7</td>
</tr>
<tr>
<td>Urinary nitrate</td>
<td>90†</td>
<td>44</td>
<td>65†</td>
<td>41</td>
<td>21</td>
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<tr>
<td>Vascular eNOS activity</td>
<td>1446†</td>
<td>631</td>
<td>923†</td>
<td>305</td>
<td>466</td>
</tr>
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<td>Urinary ET-1</td>
<td>−521</td>
<td>−51†</td>
<td>−1</td>
<td>−4</td>
<td>−40</td>
</tr>
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</table>

eNOS indicates endothelial NO synthase; ET, endothelin; HO, heme oxygenase; LTBA, leukotriene B4; ND, not determined.

*Data are expressed as percentage change compared with the apolipoprotein E−/− mouse fed a control diet.

†P<0.05 vs apolipoprotein E−/− control mice.

ApoE−/− mice. Selected phenolic acids and some polyphenols have been shown to inhibit eicosanoid pathways. It has previously been shown that quercetin and its in vivo metabolites are capable of inhibiting LTBA production in vitro in human neutrophils. (minus)-Epicatechin and related flavonoids have been shown to inhibit the synthesis of proinflammatory cytokines in vitro. Theaflavin protected against inflammation in mice by inhibiting arachidonic acid metabolism via both 5-lipoxygenase and cyclooxygenase pathways. Sesamin inhibited lipopolysaccharide-induced interleukin 6 production by suppression of the p38 mitogen-activated protein kinase signal pathway and nuclear factor-κB activation. Our results showed that quercetin and theaflavin significantly inhibited the ex vivo production of proinflammatory LTBA in the vasculature of the ApoE−/− mice and were better inhibitors of vascular LTBA production than (−)-epicatechin and sesamin (Figure 4C). Chlorogenic acid had no effect on vascular LTBA production, although it has been reported to exhibit other anti-inflammatory properties in vitro. The fact that both quercetin and theaflavin were also able to significantly inhibit lesion formation suggests that this anti-inflammatory property contributes to the antiatherogenic effect of these 2 polyphenols.

The endothelium regulates vascular tone by balancing the production of vasodilators, most important NO, and vasoconstrictors, such as ET-1. Disruption of this balance may result in endothelial dysfunction. Polyphenol consumption may help to reverse endothelial dysfunction. Oral administration of pure (−)-epicatechin to humans increased flow-mediated dilation, closely emulating the short-term vascular effects of flavonol-rich cocoa. It has been shown that quercetin and (−)-epicatechin (200 mg each) immediately augment NO status and reduce ET-1 production in healthy men. Our data suggest that quercetin and theaflavin, in particular, may improve endothelial function by increasing eNOS activity and augmenting NO production, as indicated by increased urinary nitrate excretion, in ApoE−/− mice. Quercetin and (−)-epicatechin have been shown to increase eNOS activity in endothelial cells in vitro via the inhibition of NADPH oxidase. Although quercetin and (−)-epicatechin both significantly reduced urinary ET-1, only quercetin significantly inhibited lesion size in this model. Endothelin ETA receptor blockade has been shown to inhibit atherosclerosis in ApoE−/− mice fed a high-fat diet, suggesting a role for ET-1 in this model. Unfortunately, we did not measure vascular ET-1 protein content in our study, which may have been a better marker than urinary excretion.

The attenuation of lesion formation in the quercetin-fed ApoE−/− mouse was associated with increased expression of HO-1 protein in aortic lesions. HO-1 is thought to play a key protective role in inflammatory atherosclerotic disease. Inducers of HO-1 reduce lesion size in apoE−/− mice, LDL receptor−/− mice, and rabbits. Moreover, adenovirus-mediated overexpression of HO-1 decreases lesion size in ApoE−/− mice. Several polyphenols, including curcumin and resveratrol, have been reported to induce HO-1 expression and activity in vitro, although we are not aware of any previous report showing in vivo HO-1 induction by a polyphenol in the context of atherosclerosis. The synthetic phenol probucol and its metabolites can induce HO-1 and may be the main mode of action for the protective effects of this compound against atherosclerotic disease. Interestingly, arterial induction of HO-1 can protect eNOS from oxidative inactivation, and NO can induce HO-1, so that the 2 pathways may interact with each other in providing protection against atherosclerosis by polyphenols and other compounds.

Our study showed that the extent of atherogenesis in ApoE−/− mice correlated most strongly with eNOS activity (R = −0.67, P<0.001) and urinary nitrate (R = −0.52, P<0.001), consistent with the notion that NO bioavailability plays an important role in atherogenesis. This may explain why quercetin and theaflavin protected against atherosclerosis, whereas (−)-epicatechin did not, even though the latter showed significant antioxidant actions (Table). Although some of this evidence relies on correlation data, more detailed studies will be required to establish a mechanistic link to atherogenesis.

We have shown that certain dietary polyphenols may have a range of bioactivities that contribute to the cardioprotective...
effects of fruits, vegetables, red wine, and tea. Our results indicate that key dietary polyphenols, such as quercetin and theaflavin, may act to prevent atherosclerosis through a combination of effects, including the inhibition of inflammation, the stimulation of NO, and the induction of HO-1. This study had limitations. We chose a dose of each polyphenol compound that may represent a reasonable dietary intake and added each compound to the diet at the same concentration. This may not reflect the different dietary abundance of these compounds or differences in the bioavailability between the compounds. Future studies examining a dose-response for these compounds are required.

Acknowledgments
We thank Suntory Wellness Limited, Japan, for providing sesamin and Unilever for providing theaflavin. Dr Loke thanks the University of Western Australia for an International Research Fees Scholarship.

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Disclosures
Dr Stocker holds a patent on HO-1–inducing compounds for the treatment of cardiovascular disease.

References
Specific Dietary Polyphenols Attenuate Atherosclerosis in Apolipoprotein E−Knockout Mice by Alleviating Inflammation and Endothelial Dysfunction

Wai Mun Loke, Julie M. Proudfoot, Jonathan M. Hodgson, Allan J. McKinley, Neil Hime, Maria Magat, Roland Stocker and Kevin D. Croft

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Supplement Material

Materials

(-)-Epicatechin, 2,3,4,5,6-pentafluorophenylbromide (PFPBr), adenosine triphosphate (ATP), arabic gum, bovine serum albumin (BSA), butylated hydroxytoluene (BHT), calcium ionophore, calcium chloride, chlorogenic acid, formaldehyde, glucose, Hepes, heptadecanoic acid, Kaiser's glycerine glycol, lucigenin, magnesium sulphate, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), nicotinamide adenine dinucleotide, reduced (NADH), potassium chloride, potassium phosphate monobasic, pyridine, quercetin, sodium chloride, sodium hydrogencarbonate, sodium nitrate-\(^{15}\)N, sodium nitrite-\(^{15}\)N, sodium phosphate dibasic, Sudan (IV) and toluene were purchased from Sigma Aldrich (St Louis, MO, USA); arachidonic acid from Cayman Chemical (Michigan, USA); phosphate buffered saline (PBS) from Gibco™ Invitrogen (Calsbad, CA, USA); acetone, chloroform, ethanol, hexane, methanol, n-heptane, sulfuric acid from Univar (WA, Australia). Sesamin and theaflavin were kindly provided by Suntory (Japan) and Unilever (Netherlands) respectively.

Histological Analysis of Mouse Aortas

The aorta was placed in 0.5 mL of a gum sucrose solution (15% sucrose and 1% arabic gum in water, w/w) and left overnight at 4 °C. The next day, the aortic tissue was rinsed in PBS and blotted dry before it was completely frozen in OCT compound (Tissue-Tek®) and then cryostat-sectioned (20 μm thickness) every 100 μm from the tip of the aortic sinus or from the first pair of intercostal arteries (for the thoracic aorta) for a total distance of 1200 μm using a cryostat (Leica CM3050S). The section was stained with Sudan (IV) solution (0.5 g Sudan (IV), 35 mL ethanol, 50 mL acetone, and 15 mL water),
rinsed with 80% ethanol, blotted dry and covered with a coverslip using Kaiser's glycerine glycol. The specimen was examined using light microscopy with a built-in camera under 10 x magnification (Nikon Eclipse TS100). The total aortic tissue and lesion areas were analyzed on the image collected using Nikon NIS Elements Imaging Software BR 2.30, SP2 (Build 361). Lesions were stained red. Cross-sectional lesion areas were measured at both the aortic sinus and the region immediately below the thoracic arch. The observer was blinded to treatment groups.

**Superoxide analysis**

Briefly, the fresh abdominal aortic tissue was weighed before placement in an opaque 96-well microtiter plate in PBS at pH 7.5 with 100 µM NADH followed by incubation at 37 °C under 95% O₂, 5% CO₂ for 30 minutes and luminescence measurement with a Wallac Victor-II (PerkinElmer Life Sciences) in the luminometry mode. Lucigenin at a final concentration of 5 µM was added and luminescence count recorded at 1-minute intervals for 30 minutes. The concentration of lucigenin was kept at 5 µM to limit redox-recycling which can lead to increased superoxide production. The residual aortic tissue was then homogenised in 1 mL PBS and the protein content in the homogenate was determined as previously described. The detected signal was inhibited by 90% in controls where SOD (final concentration 150 units/mL) was added to the test samples. The amount of superoxide radical ion detected was corrected for the protein content of the aortic tissues.

**Aortic Fatty Acid Composition**

For fatty acid analysis, the dried lipid extracts from the mouse aortas was heated in boiling water for 10 minutes with 2 mL 4% H₂SO₄ in methanol and 50 µL heptadecanoic
acid (internal standard, stock 1 mg/mL). The methyl esters of fatty acids were analysed by gas chromatography as previously described\textsuperscript{3}.

\textit{Ex Vivo Vascular Leukotriene B\textsubscript{4} Production}

Fresh aortic tissue was weighed before immersing in HBHS [CaCl\textsubscript{2}.2H\textsubscript{2}O (0.09 g), glucose (0.50 g), Hepes (0.06 g), KCl (0.20 g), KH\textsubscript{2}PO\textsubscript{4} (0.03 g), MgSO\textsubscript{4}.7H\textsubscript{2}O (0.10 g), NaHCO\textsubscript{3} (0.18 g), NaCl (4.00 g), Na\textsubscript{2}HPO\textsubscript{4} (0.02 g) and BSA (0.50 g) in pure water (500 mL); pH 7.4]. The tissue was then incubated with ATP (final concentration 2 mM), Ca\textsuperscript{2+} ionophore A\textsubscript{23187} (final concentration 2.5 µg/mL) and arachidonic acid (final concentration 10 µM) at 37 °C for 30 minutes. The supernatant was analysed for LTB\textsubscript{4} using a specific LTB\textsubscript{4} enzyme immunoassay (EIA) kit (Cayman Chemical, Michigan, USA). The residual aortic tissue was then homogenised in 1 mL PBS and the protein content in the homogenate was determined as previously described\textsuperscript{2}.
Table 1. Calculated nutritional parameters of AIN93M mouse diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>13.6%</td>
</tr>
<tr>
<td>Total fat</td>
<td>4%</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>4.7%</td>
</tr>
<tr>
<td>Acid detergent fibre</td>
<td>4.7%</td>
</tr>
<tr>
<td>Digestive energy</td>
<td>15.1 MJ/kg</td>
</tr>
</tbody>
</table>

Table 2. Mean cholesterol concentrations in the plasma from C57BL/6J and ApoE⁻/⁻ mice after 10 weeks (16 weeks of age) and 20 weeks (26 weeks of age) of different dietary treatments.

<table>
<thead>
<tr>
<th>Mice/Treatment</th>
<th>Week 16 (n = 5 per group)</th>
<th>Week 26 (n = 20 per group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range (Min, Max)</td>
</tr>
<tr>
<td>C57BL/6J Control</td>
<td>2.82 ± 1.90*</td>
<td>1.46, 6.12</td>
</tr>
<tr>
<td>ApoE Control</td>
<td>8.10 ± 1.98</td>
<td>3.55, 14.37</td>
</tr>
<tr>
<td>ApoE Quercetin</td>
<td>7.58 ± 4.84</td>
<td>1.62, 14.22</td>
</tr>
<tr>
<td>ApoE Epicatechin</td>
<td>9.39 ± 6.30</td>
<td>1.94, 19.38</td>
</tr>
<tr>
<td>ApoE Theaflavin</td>
<td>8.52 ± 1.27</td>
<td>6.45, 9.83</td>
</tr>
<tr>
<td>ApoE Sesamin</td>
<td>8.68 ± 7.54</td>
<td>2.12, 18.84</td>
</tr>
<tr>
<td>ApoE Chlorogenic acid</td>
<td>10.17 ± 3.27</td>
<td>5.68, 13.63</td>
</tr>
</tbody>
</table>

*p < 0.05 vs ApoE/ Control mice at respective time point.
Table 3. Mean nitrite and (B) nitrate concentrations in the urine from C57BL/6J and ApoE<sup>-/-</sup> mice after 20 weeks (26 weeks of age) of different dietary treatments.

<table>
<thead>
<tr>
<th>Mice/ Treatment</th>
<th>Urinary nitrite†</th>
<th>Urinary nitrate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J Control</td>
<td>0.64±0.14</td>
<td>15.45±1.38*</td>
</tr>
<tr>
<td>ApoE Control</td>
<td>0.41±0.08</td>
<td>8.20±0.92</td>
</tr>
<tr>
<td>ApoE Quercetin</td>
<td>0.65±0.18</td>
<td>15.58±1.32*</td>
</tr>
<tr>
<td>ApoE Epicatechin</td>
<td>0.64±0.11</td>
<td>11.80±0.44</td>
</tr>
<tr>
<td>ApoE Theaflavin</td>
<td>0.32±0.04</td>
<td>13.54±1.41*</td>
</tr>
<tr>
<td>ApoE Sesamin</td>
<td>0.60±0.16</td>
<td>11.54±1.52</td>
</tr>
<tr>
<td>ApoE Chlorogenic acid</td>
<td>0.37±0.02</td>
<td>9.92±0.88</td>
</tr>
</tbody>
</table>

† expressed as mean±SEM in µmol/mmol creatinine (n = 5 per group).
* p < 0.05 vs ApoE/ Control mice.
Table 4. The degree of correlation between lesion area (in the aortic sinus and thoracic aorta) and the measured biochemical markers

<table>
<thead>
<tr>
<th>Biochemical Markers</th>
<th>Correlation Factor, R</th>
<th>Significance, P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) Aortic sinus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic F(_2)-isoprostanes</td>
<td>0.289*</td>
<td>0.000</td>
</tr>
<tr>
<td>Aortic superoxide</td>
<td>0.046</td>
<td>0.302</td>
</tr>
<tr>
<td>Aortic LTB(_4)</td>
<td>0.105</td>
<td>0.113</td>
</tr>
<tr>
<td>Plasma P-Selectin</td>
<td>0.079</td>
<td>0.183</td>
</tr>
<tr>
<td>Aortic eNOS activity</td>
<td>-0.670*</td>
<td>0.000</td>
</tr>
<tr>
<td>Urinary ET-1</td>
<td>0.110</td>
<td>0.265</td>
</tr>
<tr>
<td>Urinary NO(_2)^-</td>
<td>-0.052</td>
<td>0.384</td>
</tr>
<tr>
<td>Urinary NO(_3)^-</td>
<td>-0.52*</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>(B) Thoracic aorta</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic F(_2)-isoprostanes</td>
<td>0.219*</td>
<td>0.006</td>
</tr>
<tr>
<td>Aortic superoxide</td>
<td>0.195*</td>
<td>0.013</td>
</tr>
<tr>
<td>Aortic LTB(_4)</td>
<td>0.214*</td>
<td>0.007</td>
</tr>
<tr>
<td>Plasma P-Selectin</td>
<td>0.080</td>
<td>0.179</td>
</tr>
<tr>
<td>Aortic eNOS activity</td>
<td>-0.381*</td>
<td>0.012</td>
</tr>
<tr>
<td>Urinary ET-1</td>
<td>0.196</td>
<td>0.129</td>
</tr>
<tr>
<td>Urinary NO(_2)^-</td>
<td>0.118</td>
<td>0.251</td>
</tr>
<tr>
<td>Urinary NO(_3)^-</td>
<td>-0.375*</td>
<td>0.013</td>
</tr>
</tbody>
</table>

*Correlation is significant at 0.05 level
Figure 1: Thoracic aorta transverse sections from (A) C57BL/6J, (B) ApoE control, (C) ApoE quercetin and (D) ApoE theaflavin at 26 weeks of age under 10x magnification after Sudan(IV) staining.
Figure 2

Cholesterol distribution in lipid classes separated from mouse plasma by FPLC
References

