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Quercetin up-regulates paraoxonase 1 gene expression with concomitant protection against LDL oxidation

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ABSTRACT

Paraoxonase 1 (PON1) protects the oxidative modification of low-density lipoprotein (LDL) and is a major anti-atherosclerotic protein component of high-density lipoprotein (HDL). Quercetin, a ubiquitous plant flavonoid, has been shown to have a number of bioactivities and may offer a variety of potential therapeutic uses. We explored the roles of quercetin in the regulation of PON1 expression, serum and liver activity and protective capacity of HDL against LDL oxidation in rats. Compared to the pair-fed control group, feeding quercetin (10 mg/L) in the liquid diet for 4 weeks increased (a) hepatic expression of PON1 by 35% ($p < 0.01$), (b) serum and liver PON1 activities by 29% ($p < 0.05$) and 57% ($p < 0.01$), respectively, and (c) serum homocysteine thiolactonase (HCTL) activity by 23% ($p < 0.05$). Correspondingly, the lag time of low-density lipoprotein (LDL) oxidation was increased by >3-fold ($p < 0.001$) with plasma HDL from quercetin-fed group compared to the HDL from control group. Our data suggest that quercetin has antiatherogenic effect by up regulating PON1 gene expression and its protective capacity against LDL oxidation.

The paraoxonase 1 (PON1) is a calcium-dependent ester hydrolase tightly associated with high-density lipoprotein (HDL) particles [1,2]. Experimental evidences have shown that PON1 functions as an antioxidative enzyme that inhibits the oxidation of low-density lipoprotein (LDL) [3–5], which prevents the development of atherosclerosis [6,7]. It also hydrolyses homocysteine thiolactone to homocysteine, which alters the proteins in the arterial wall [8]. PON1 knock-out mice are more susceptible to atherosclerosis than wild-type littermates and clinical studies have linked PON1 to the prevention of cardiovascular disease [9,10].

Even though the PON1 gene polymorphism may play certain roles in the development of coronary artery disease (CAD) [11–13], its phenotypic expression is more closely related to CAD [14]. It is known that PON1 inhibits cholesterol biosynthesis [15] and stimulates HDL-mediated cholesterol efflux from macrophages [3,16–18] thus preventing CAD development. Therefore, it is more important to investigate possible dietary supplements that could influence PON1 activity and/or its expression. Quercetin, an ubiquitous flavonoid present in all fruits and vegetables, has been shown to have free radical-scavenging property and to reduce low-density lipoprotein (LDL) oxidation [19,20], and thus may benefit with respect to cardiovascular disease (CVD) and certain cancers [21]. In this communication, we demonstrate that dietary quercetin significantly up regulates PON1 expression, activity and its HCTL activity and significantly increases protective capacity of HDL against LDL oxidation.

Materials and methods

Animal feeding. Male Wistar rats weighing between 130 and 150 g. were purchased from Charles River Laboratories, Inc. (Wilmington, MA). The rats were housed in groups of 2 per cage in plastic cages (40 × 24 × 18 cm), in a temperature-controlled room at 25 °C with 12-h light: dark cycles. All rats were fed a pelleted commercial diet (Purina Rodent Chow, #500, TMI Nutrition, St. Louis, MO) for 1 week after arrival. They were then randomly divided into 2 groups (6 animals/group) and pair-fed the control Lieber–DeCarli liquid diet [22] or quercetin liquid diet for 4 weeks. The concentration of quercetin used in the diet was 10 mg/liter, which was isoenergetic to control Lieber–DeCarli diet. At the end of the experimental period, all the animals in each group were exanguinated via abdominal aorta under pentobarbital anesthesia (50 mg/kg, ip), and the blood serum and livers were saved for further analyses as described below:

Protein extracts from liver. Livers from control and quercetin-fed rats were harvested and washed three times with ice-cold phosphate-buffered saline (PBS). The tissues were then homogenized

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in 10 mM HEPES buffer (pH 7.5) containing 1 mM EDTA, 20% glycerol, 10 mM NaCl, and 1 μg/ml each of the following protease inhibitors: phenylmethylsulfonyl fluoride, aprotinin, and leupeptin. The nuclei were removed by centrifugation at 2000 *g* for 5 min at 4 C followed by centrifugation at 100,000 *g* for 30 min at 4 °C. The resulting microsomal pellet was resuspended in a suitable volume of the homogenization buffer for the determination of PON1 activity. The total protein concentration of the extract was determined by Bradford method (Bio-Rad, Hercules, CA).

RNA isolation. The total RNA was isolated from each liver using the Tri-Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. Adequate measures were undertaken to ensure high quality of RNA extracted in the samples. Briefly, 100 mg of each liver tissue was homogenized in 1 ml of Tri-Reagent. The homogenates were left for 5 min at room temperature followed by addition of 0.2 ml of BCP phase separation reagent, shaken vigorously for 5 s and again left at room temperature for additional 15 min. After centrifugation (12,000 *g* for 20 min) at 4 °C, the upper aqueous phase was carefully pipetted out into a sterile tube. The RNA was precipitated by addition of 0.5 ml of isopropanol and incubated at room temperature for 5–10 min. RNA was pelleted by centrifuging again at 12,000 *g* at 4 °C for 15 min. The precipitated RNA was washed in 70% ethanol, briefly air-dried, and then solubilized in Formazol (Molecular Research Center, Cincinnati, OH). Total RNA concentrations were measured by OD reading using SpectroMAX 190 (Molecular Devices Co., Sunnyvale, CA). The purity and concentration of total RNA samples were examined by determining the A_{260}/A_{280} ratio. Isolated RNA was used immediately or stored at −80 °C until use.

Serum and liver PON1 activity measurement. Serum and liver PON1 enzyme activity was determined with paraoxon (Sigma–Aldrich Inc., St. Louis, MO) as the substrate. PON1 activity was measured as described by us previously [23] in an aliquot of serum or the microsomal suspension from liver based on the initial velocity of *p*-nitrophenol production at 37 °C and continuously monitored at 405 nm using SpectroMAX 190 (Molecular Devices, Sunnyvale, CA). 10 μl of serum or microsomal suspension was added into 990 μl 20 mM Tris–HCl buffer, pH 7.8 containing 2 mM $CaCl_2$ and 5 mM of paraoxon. Results were expressed as IU. One unit of international enzyme activity was equal to 1 nmol of paraoxon hydrolyzed per minute per ml of serum or per g equivalent of liver microsomes.

Measurement of serum HCTL activity. Serum HCTL activity was determined essentially as described by us previously [24]. Briefly, [^{35}S]-homocysteine thiolactone was synthesized from [^{35}S]-methionine based on prior methods [25–27]. A 50 μl aliquot of the serum was incubated with purified [^{35}S]-homocysteine thiolactone (5 mmol/L) in 0.1 mol/L potassium phosphate-HEPES buffer (pH 7.4) and 2 mmol/L $CaCl_2$ for 1 h, and the radioactivity in labeled homocysteine formed was determined by thin-layer chromatography. HCTL activity is expressed as nanomoles of homocysteine formed per ml of serum per minute under the above assay conditions.

HDL protection assay on the oxidation of LDL by $CuSO_4$. HDL was isolated from each serum sample according to the conventional methods [28,29]. The ability of HDL to protect against LDL oxidation by $CuSO_4$, as indicated by the increase in lag phase, was measured spectrophotometrically at 234 nm at 37 °C using SpectraMAX 190 (Molecular Devices, Sunnyvale, CA) as described by us previously [24]. Each assay (1 ml final volume) contained 100 μg apolipoprotein A1 equivalent of HDL fraction, 100 μg/ml LDL protein, and 10 μM $CuSO_4$ in PBS (pH 7.4). Immediately after $CuSO_4$ addition, 100 μl aliquots of the assay were distributed into 4 wells of a quartz 96-well micro-titer plate, and lipid peroxidation occurrence (i.e., LDL oxidation) was monitored at 234 nm for 5 h. For each quercetin-fed and control HDL sample, a negative control assay was carried out without $CuSO_4$. LDL oxidation protection was assessed by measuring the length of the lag phase (i.e., lag time) of the oxidation kinetics curves.

Real-time RT-PCR. cDNA templates for use in real-time PCR were synthesized from 5 μg of total RNA by in vitro transcription in 20 μl reaction containing 0.5 μg Oligo (dT), 10 μM dNTPs and 1 u of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) at 42 °C for 50 min. Typical real-time PCR reaction mixture included the same amount of cDNA templates from RT, 10 pM of each primers, 25 μl iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and sterile water in a reaction volume of 50 μl. The PCR conditions were the following: 3 min at 95 °C follow by 40 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The primer pairs for rat PON1 are: forward primer 5′-TGCTGGCTCACAAGATTCA C-3′ and reverse primer 5′-TTCCTTTGTACACAGCAGCG-3. This primer pair was first tested by regular PCR to be highly effective and specific for amplification. Actin was used as the standard housekeeping gene. Ratios of PON1 and actin expression levels were calculated by subtracting the threshold cycle number (Ct) of the target gene (PON1) from the Ct of actin and raising 2 to the power of this difference. Ct values are defined as the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold. Target gene expressions are expressed relative to actin expression.

Statistical analysis. Data are presented as the Mean ± SD. Statistical significance was determined by one-way ANOVA test followed by the Tukey's test.

Results

Effect of quercetin on serum and liver PON1 activity

To investigate whether quercetin influenced PON1 activity in vivo as observed with cells in vitro [21], serum and liver PON1 activities were measured in rats pair-fed for 4 weeks from the control diet and the same diet supplemented with quercetin at a concentration of 10 mg/L. The results are shown in Table 1. Compared to pair-fed control group, quercetin-fed group exhibited increased serum PON1 activity by 29% ($p < 0.05$) and liver PON1 activity by 57% ($p < 0.01$).

Effect of quercetin on serum HCTL activity

To investigate whether quercetin influenced serum HCTL activity in vivo, serum HCTL activity was measured in rats pair-fed for 4 weeks from the control and the same diet supplemented with quercetin at a concentration of 10 mg/L. The results are shown in Table 2.

Table 1
Influence of quercetin feeding on serum and liver PON1 activity. Serum and liver PON1 activities were determined from both control and quercetin-fed groups as described in the Materials and Methods section. Each value is the Mean ± SD of 6 independent determinations.

Group	Serum PON1 activity (nmol paraoxon hydrolyzed/ml/min)	*P* value	Liver PON1 activity (nmol paraoxon hydrolyzed/g/min	*P* value
Control	50.13 ± 3.5		41.9 ± 13.7	
Quercetin	64.53 ± 9.1	<0.05	65.69 ± 8.2	<0.01

Table 2
Influence of quercetin feeding on serum HCTL activity. Serum HCTL activity was determined in an aliquot of the serum of each animal from both control and quercetin-fed groups as described in the Materials and methods section. Each value is the Mean ± SD of 6 independent determinations.

Group	Serum HCTL activity (nmol HCTL hydrolyzed/ml/min)	*P* value
Control	6.97 ± 0.52	
Quercetin	8.57 ± 0.61	<0.05