JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Quercetin and Its In Vivo Metabolites Inhibit Neutrophil-Mediated Low-Density Lipoprotein Oxidation

WAI MUN LOKE,[†,‡] JULIE M. PROUDFOOT,[†] ALLAN J. MCKINLEY,[‡] PAUL W. NEEDS,[§] PAUL A. KROON,[§] JONATHAN M. HODGSON,[†] AND KEVIN D. CROFT[†,*]

School of Medicine and Pharmacology, University of Western Australia, Royal Perth Hospital Unit, Medical Research Foundation Building, Level 4, 50 Murray Street, Perth, WA 6000, Australia, School of Biomedical, Biochemical and Chemical Sciences, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia, and Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, United Kingdom

This study examined the effects of metabolic transformation of the common dietary flavonoid, quercetin, on its ability to protect low-density lipoprotein (LDL) from neutrophil-mediated modification. Quercetin was shown to be effective in protecting LDL against neutrophil-mediated modification at physiological concentrations (1 μM) and appears to act by inhibiting myeloperoxidase (MPO)-catalyzed oxidation (IC_{50} = 1.0 μM). Quercetin was also shown to protect against radical-induced [2,2′-azobis(2-methylpropionamidine)dihydrochloride] oxidation (IC_{50} = 1.5 μM). Studies of structure−activity relationships showed that methylation at the 3′-position or glucuronidation at the 3-position did not significantly affect inhibition by quercetin of the MPO activity, but conjugations at both positions significantly reduce its activity. Our results suggest that the common dietary flavonoid, quercetin, and some of its major in vivo metabolites are potential inhibitors of MPO at physiological concentrations. Dietary flavonoids that could modify MPO activity could protect lipoproteins from damage in chronic inflammatory states such as cardiovascular disease.

KEYWORDS: Antioxidant; myeloperoxidase; metabolic transformation; flavonoid structure

INTRODUCTION

Oxidative damage to low-density lipoprotein (LDL) has been proposed as a critical step in the development of atherosclerosis (*1*). Studies with human populations have demonstrated that circulating oxidized LDL is associated with preclinical atherosclerosis, coronary arterial atherosclerosis, acute coronary syndrome, and vulnerable plaques (*2*). Circulating oxidized LDL is believed to be a useful marker for identifying patients with coronary heart disease (*2*). Nitrotyrosine, a marker of LDL protein damage, is nearly 100-fold higher in LDL recovered from human atherosclerotic aorta than that in circulating LDL from healthy donors (*3*).

One pathway for LDL modification in vivo is through reactive species generated by myeloperoxidase (MPO) (*4*). MPO has been suggested as a physiological catalyst for in vivo LDL modification in studies using monocytes and neutrophils isolated from humans (*5*). Previous reports of elevated plasma MPO in early adverse cardiac events (*6*), in acute coronary syndrome (*7*), and after acute myocardial infarction (*8*) provide further evidence that MPO may contribute to cardiovascular disease. More recently, MPO has been implicated in the production of cyanate, which can cause carbamylation of proteins (*4*).

Population studies suggest that dietary flavonoids may provide protection against cardiovascular disease (*9*). Recent controlled intervention trials report that flavonoid-rich food can improve endothelial and platelet function and reduce blood pressure in humans (*10*) and may inhibit the development of atherosclerosis in animal models (*11*). Because oxidative stress has been implicated in atherosclerosis and cardiovascular disease, one of the main properties of flavonoids thought to explain their health benefit is their antioxidant activity (*12*). However, the results of intervention studies have yielded conflicting results (*13*). This may be due to variations in the absorption and metabolism of flavonoids, which may alter antioxidant activity as well as other biological activities (*14*). In particular, there is doubt about the interpretation of in vitro studies of antioxidant activity where issues of bioavailability and metabolic transformation have not been considered (*15*).

* To whom correspondence should be addressed. Tel: +61892240275. Fax: +61892240246. E-mail: Kevin.croft@uwa.edu.au.

[†] School of Medicine and Pharmacology, University of Western Australia.

[‡] School of Biomedical, Biomolecular and Chemical Sciences, University of Western Australia.

[§] Institute of Food Research.

10.1021/jf8003042 CCC: $40.75
Published on Web 05/03/2008

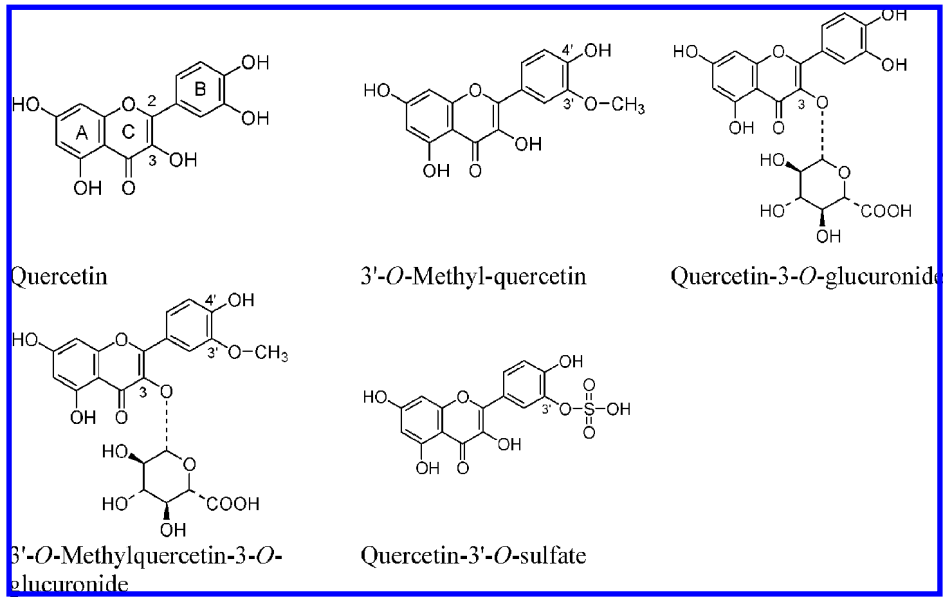

Figure 1. Structures of quercetin and its metabolites present in human circulation.

To address the issue of the effect of metabolic transformation on flavonoid bioactivity, we have compared the ability of quercetin and its major in vivo metabolites to inhibit LDL modification by human neutrophils, a rich source of MPO. We have also examined the structural features important for antioxidant activity by comparing quercetin and its major metabolites (**Figure 1**) as well as other structurally related flavonoids. We found that structural modification of quercetin due to metabolic transformation can have an effect on bioactivity. We also found that quercetin and some of its metabolites protect LDL from MPO-mediated modification in vitro.

MATERIALS AND METHODS

Chemicals and Reagents. Bovine serum albumin (BSA), calcium chloride, guaiacol, sodium acetate, quercetin, sodium phosphate dibasic, sodium phosphate monobasic, sodium bromide, sodium chloride, sodium hydrogencarbonate, luteolin, kaempferol, taxifolin, butylated hydroxytoluene, hydrogen peroxide (50% by volume), phorbol 12-myristate 13-acetate (PMA), methane sulfonic acid, phenol, benzoic acid, superoxide dismutase (SOD), ferricytochrome *c*, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), and diethylenetriamine penta-acetic acid were purchased from Sigma Aldrich (St. Louis, MO); acetonitrile, magnesium sulfate, and sulfuric acid were from Univar (WA, Australia); ficoll-paque was from GE Healthcare (Uppsala, Sweden); Hanks' balanced salt solution (HBSS), phosphate-buffered saline (PBS), heat-inactivated fetal calf serum (HIFCS), and RPMI 1640 were from Gibco Invitrogen (Carlsbad, CA); dextran 500 was from Amersham Biosciences (Uppsala, Sweden); glucose and potassium phosphate monobasic were from Merck (VIC, Australia), and methanol and ethanol were from Mallinckrodt (NJ). 3′-*O*-Methylquercetin, quercetin-3′-*O*-sulfate, quercetin-3-*O*-glucuronide, and 3′-*O*-methylquercetin-3-*O*-glucuronide were synthesized as previously described (*16*).

AAPH-Induced and Neutrophil-Mediated Modification of LDL. Neutrophils were isolated from the neutrophil/erythrocyte pellet after Ficoll−Paque gradient centrifugation and dextran sedimentation of red cells as previously described (*17*). Cell viability was assessed using trypan blue exclusion and was typically >98%. LDL was isolated immediately from fresh plasma by density gradient ultracentrifugation, and its protein concentration was determined as previously described (*18*). LDL (final protein concentration, 0.1 mg/mL) was incubated at 37 °C in the presence of either PMA-activated peripheral neutrophils (5×10^6 cells/mL HBSS; final PMA concentration, 200 nM) or AAPH (final concentration, 5 mM). The supernatant from the cell suspension was collected at regular time intervals and stored at −80 °C before F_2-isoprostane and 3-chlorotyrosine analyses. LDLs incubated with PMA-activated cells were used as positive controls, while LDLs incubated with untreated cells without PMA activation served as negative controls.

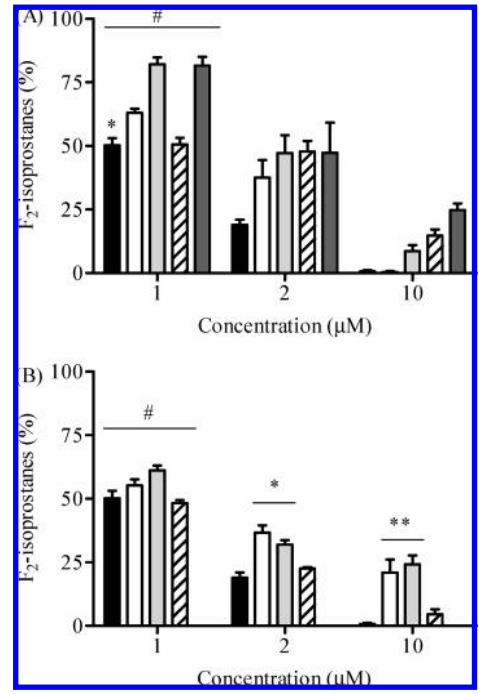

Figure 2. (**A**) Dose-dependent effects of quercetin (Q, black bar), 3′-*O*-methylquercetin (MQ, white bar), quercetin-3′-*O*-sulfate (QS, light gray bar), quercetin-3-*O*-glucuronide (QG, striped bar), and 3′-*O*-methylquercetin-3-*O*-glucuronide (MQG, dark gray bar) on the production of F_2-isoprostanes by PMA-activated neutrophils ($n = 3$). $^*p < 0.05$ vs quercetin-3′-*O*-sulfate and 3′-*O*-methylquercetin-3-*O*-glucuronide at 1 μM concentration. $^\#p < 0.05$ vs the positive control. (**B**) Dose-dependent effects of quercetin (Q, black bar), luteolin (L, white bar), kaempferol (K, light gray bar), and taxifolin (T, striped bar) on the production of F_2-isoprostanes by PMA-activated neutrophils ($n = 3$). $^*p < 0.05$ vs quercetin at 2 μM concentration. $^{**}p < 0.05$ vs quercetin at 10 μM concentration. $^\#p < 0.05$ vs the positive control.

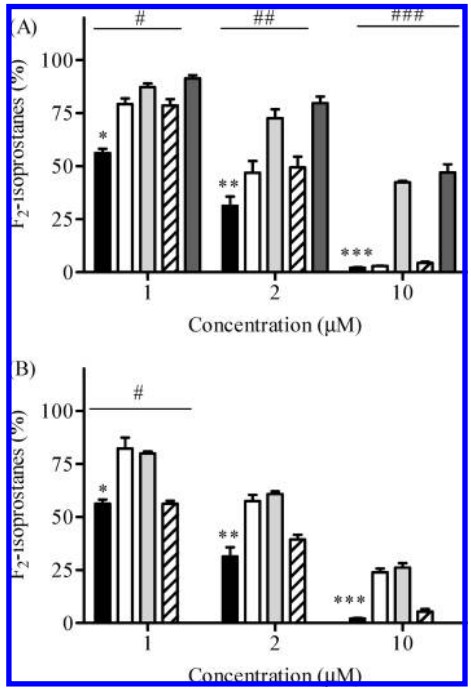

Figure 3. (**A**) Dose-dependent effects of quercetin (Q, black bar), 3′-*O*-methylquercetin (MQ, white bar), quercetin-3′-*O*-sulfate (QS, light gray bar), quercetin-3-*O*-glucuronide (QG, striped bar), and 3′-*O*-methylquercetin-3-*O*-glucuronide (MQG, dark gray bar) on the production of F_2-isoprostanes after AAPH treatment of LDL ($n = 3$). *$p < 0.05$ vs all metabolites at 1 μM concentration. **$p < 0.05$ vs quercetin-3′-*O*-sulfate and 3′-*O*-methylquercetin-3-*O*-glucuronide at 2 μM concentration. ***$p < 0.05$ vs quercetin-3′-*O*-sulfate and 3′-*O*-methylquercetin-3-*O*-glucuronide at 10 μM concentration. $^{\#}p < 0.05$ vs the positive control at 1 μM concentration. $^{\#\#}p < 0.05$ vs the positive control at 2 μM concentration. $^{\#\#\#}p < 0.05$ vs the positive control at 10 μM concentration. (**B**) Dose-dependent effects of quercetin (Q, black bar), luteolin (L, white bar), kaempferol (K, light gray bar), and taxifolin (T, striped bar) on the production of F_2-isoprostanes after AAPH treatment of LDL ($n = 3$). *$p < 0.05$ vs luteolin and kaempferol at 1 μM concentration. **$p < 0.05$ vs luteolin and kaempferol at 2 μM concentration. ***$p < 0.05$ vs luteolin and kaempferol at 10 μM concentration. $^{\#}p < 0.05$ vs the positive control.

Measurement of Inhibition of Lipoprotein Oxidation. The antioxidant ability of each compound (quercetin, 3′-*O*-methylquercetin, quercetin-3′-*O*-sulfate, quercetin-3-*O*-glucuronide, 3′-*O*-methylquercetin-3-*O*-glucuronide, luteolin, kaempferol, and taxifolin) to inhibit AAPH-induced or neutrophil-mediated LDL oxidation was measured by the formation of F_2-isoprostanes (stable marker of lipid peroxidation) quantitated by gas chromatography−mass spectrometry (GC-MS) (*19*). Briefly, the test compound (final concentrations: 1, 2, and 10 μM) was added to the LDL and neutrophils (prior to PMA stimulation) or AAPH addition. Aliquots of the mixture were analyzed for lipid peroxidation products at specific time points up to 3 h and compared to control incubations without the addition of test compounds. LDL oxidation by neutrophils was quantified by measuring the amount of 15-F_{2t}-isoprostanes produced after 120 min, calculated relative to an internal standard (15-F_{2t}-isoprostanes-d_4). The intra- and interassay coefficients of variation (CV) for this assay were 8 ($n = 8$) and 5.6% ($n = 3$).

Measurement of Inhibition of Lipoprotein Chlorination. Inhibition of lipoprotein chlorination by quercetin, its metabolites, and its structural analogues, luteolin, kaempferol, and taxifolin was determined by measuring 3-chlorotyrosine production (*20*). Briefly, the sample was extracted with acetone, and the residue containing total proteins was hydrolyzed with methane sulfonic acid (6 M with 1% phenol and 1% benzoic acid) at 50 °C for 12 h. The hydrolysate was dissolved in the high-performance liquid chromatography (HPLC) mobile phase [20 mM sodium phosphate buffer, pH 3.0, with 5% (v/v) methanol], and an aliquot (20 μL) of the supernatant was injected into the HPLC-ECD system. Chromatography was performed on a 15 mm × 5 mm i.d., 5 micron, LiChrospher 100 RP-18 reverse phase column (Agilent Technologies) at a flow rate of 1 mL/min using a Hewlett-Packard Series 1100 HPLC. The voltage of the analytical cell was set at 550 mV. Results were obtained with within and between assay reproducibility of 7 ($n = 10$) and 3.5% ($n = 3$), respectively.

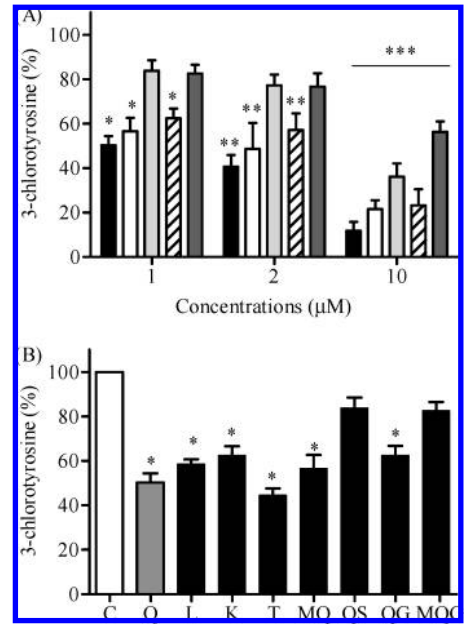

Figure 4. (**A**) Dose-dependent effects of quercetin (Q, black bar), 3′-*O*-methylquercetin (MQ, white bar), quercetin-3′-*O*-sulfate (QS, light gray bar), quercetin-3-*O*-glucuronide (QG, striped bar), and 3′-*O*-methylquercetin-3-*O*-glucuronide (MQG, dark gray bar) on the production of 3-chlorotyrosine by PMA-activated neutrophils ($n = 3$). *$p < 0.05$ vs the positive control at 1 μM concentration. **$p < 0.05$ vs the positive control at 2 μM concentration. ***$p < 0.05$ vs the positive control at 10 μM concentration. (**B**) 3-Chlorotyrosine production by PMA-activated neutrophils in the absence (C) or presence of quercetin (Q), luteolin (L), kaempferol (K), taxifolin (T), 3′-*O*-methylquercetin (MQ), quercetin-3′-*O*-sulfate (QS), quercetin-3-*O*-glucuronide (QG), and 3′-*O*-methylquercetin-3-*O*-glucuronide (MQG) (1 μM) ($n = 3$). *$p < 0.001$ vs control.

Measurement of Inhibition of Functional MPO Activity. To test the effect of quercetin, its metabolites, and its structural analogues, luteolin, kaempferol, and taxifolin, on MPO activity in peripheral neutrophils, freshly isolated cells (1×10^6 cells/mL in HBSS) were incubated with these compounds (2 μM) for 5 min at 37 °C before the incubate was removed. The cells were resuspended on fresh HBSS and lysed by sonication. Functional MPO activity was determined by measuring its catalytic action on the oxidation of guaiacol in the presence of hydrogen peroxide as described previously (*21*). The intra- and interassay CV of the results obtained were 1.3 ($n = 6$) and 2.9% ($n = 3$), respectively.

Measurement of Inhibition of NADPH Oxidase Activity. The effects of quercetin, its metabolites, and its structural analogues, luteolin, kaempferol, and taxifolin, on NADPH oxidase activity in peripheral neutrophils was investigated by measuring superoxide radical ion as described (*22*). Freshly isolated neutrophils (1×10^6 cells/mL) in HBSS were incubated with the compounds (1, 2, and 10 μM) for 5 min at 37 °C before PMA was added (final concentration, 200 nM). Superoxide production was determined by measuring the reduction of cytochrome *c* (final concentration, 100 μM) in the presence and absence of SOD (final concentration, 150 units/mL). The intra- and interassay CV of the results obtained were 3 ($n = 8$) and 2.4% ($n = 3$).

Statistical Analysis of Results. Statistical analysis of results was performed using SPSS version 11.5. One-way analysis of variance (ANOVA) (*23*) and Bonferroni posthoc analyses were performed on specific concentration points. The results analyzed were considered

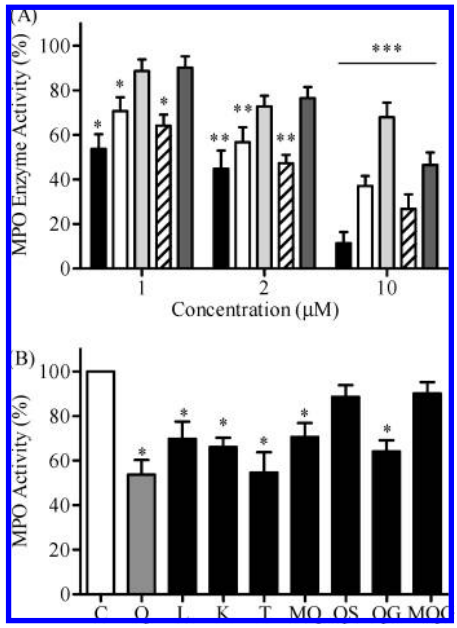

Figure 5. (**A**) Dose-dependent inhibition of functional MPO enzyme activity in lysed PMA-activated neutrophils by quercetin (Q, black bar), 3′-*O*-methylquercetin (MQ, white bar), quercetin-3′-*O*-sulfate (QS, light gray bar), quercetin-3-*O*-glucuronide (QG, striped bar), and 3′-*O*-methylquercetin-3-*O*-glucuronide (MQG, dark gray bar) ($n = 3$). *$p < 0.05$ vs the positive control at 1 μM concentration. **$p < 0.05$ vs the positive control at 2 μM concentration. ***$p < 0.05$ vs the positive control at 10 μM concentration. (**B**) Functional MPO enzyme activity in lysed PMA-activated neutrophils in the absence (C) or presence of quercetin (Q), luteolin (L), kaempferol (K), taxifolin (T), 3′-*O*-methylquercetin (MQ), quercetin-3′-*O*-sulfate (QS), quercetin-3-*O*-glucuronide (QG), and 3′-*O*-methylquercetin-3-*O*-glucuronide (MQG) (1 μM) ($n = 3$). *$p < 0.05$ vs all except quercetin-3′-*O*-sulfate and 3′-*O*-methylquercetin-3-*O*-glucuronide.

significantly different if p value ≤ 0.05 based on 95% confidence interval. Error bars in all of the figures were presented as standard error of means (SEM).

RESULTS

Effects on Lipid Peroxidation. Neutrophils did not produce F_2-isoprostanes from LDL over 120 min in the absence of PMA. LDL incubated with PMA-activated neutrophils produced significant amounts of F_2-isoprostanes up to 10-fold the initial level over the 120 min period (mean ± SEM: 145.5 ± 2.5 vs 1385.7 ± 3.2 pg/mL, $p < 0.001$). Quercetin and its metabolites exerted dose-dependent inhibition of F_2-isoprostane formation (**Figure 2A**). Quercetin protected LDL against neutrophil-mediated lipid peroxidation with an IC_{50} of approximately 1 μM, while the IC_{50} for its metabolites ranged from 2 to 4 μM. All of the metabolites had significantly lower inhibitory activities ($p < 0.05$ by ANOVA analysis of the area under dose−response curve) as compared to the parent molecule. Quercetin and its metabolites were effective in inhibiting lipid peroxidation at concentrations ranging from 1 to 10 μM (**Figure 2A**). At the realistic physiological concentration (1 μM), quercetin and all its major in vivo metabolites showed significant protection against lipid peroxidation ($p < 0.05$ vs positive control) (**Figure 2A**). Quercetin and quercetin-3-*O*-glucuronide were significantly more effective in reducing lipid peroxidation ($p < 0.05$) as compared to the other metabolites (**Figure 2A**). There was no significant difference in activity between quercetin and its metabolites at 2 μM concentration, even though the metabolites appeared to have diminished effects at both concentrations (**Figure 2A**). Quercetin and its metabolites exhibited dose-dependent inhibition of AAPH-initiated LDL peroxidation (**Figure 3A**), with an IC_{50} value of approximately 1.5 μM for quercetin and IC_{50} values ranging from 2 to 10 μM for its metabolites. At 1 μM concentration, quercetin, 3′-*O*-methylquercetin, quercetin-3′-*O*-sulfate, and quercetin-3-*O*-glucuronide offered significant protection against AAPH-initiated LDL oxidation ($p < 0.05$ vs positive control) (**Figure 3A**). Quercetin, 3′-*O*-methylquercetin, and quercetin-3-*O*-glucuronide were significantly more effective ($p < 0.05$) as compared to quercetin-3′-*O*-sulfate and 3′-*O*-methylquercetin-3-*O*-glucuronide (**Figure 3A**). When the structural analogues of quercetin, luteolin, kaempferol, and taxifolin were tested, all three significantly inhibited LDL oxidation ($p < 0.05$) (**Figure 2B**). Luteolin and kaempferol showed significantly less inhibition of neutrophil-mediated oxidation of LDL than quercetin ($p < 0.05$) at 2 and 10 μM concentrations, whereas only kaempferol was significantly less active than quercetin at a 1 μM concentration. Taxifolin was not significantly different to quercetin (**Figure 2B**). Similar trends in activity were observed during the AAPH oxidation of LDL, with all, except taxifolin, showing significantly diminished activity ($p < 0.05$) as compared to quercetin (**Figure 3B**).

Effects on LDL Protein Modification. MPO is an enzyme in neutrophils producing hypochlorous acid, which reacts with tyrosine residues in proteins to form 3-chlorotyrosine. Activated neutrophils produced at least 10-fold more LDL 3-chlorotyrosine as compared to unstimulated cells (mean ± SE.: 9.80 ± 0.55 vs 0.87 ± 0.05 μM, $p < 0.001$). Quercetin and its metabolites exhibited dose-dependent inhibition of 3-chlorotyrosine formation (**Figure 4A**). Quercetin suppressed tyrosine chlorination, with an IC_{50} value of approximately 1 μM, while its metabolites showed diminished activity with IC_{50} values ranging from 2 to 10 μM. However, the reduction in activity reached significance only when quercetin-3′-*O*-sulfate and 3′-*O*-methylquercetin-3-*O*-glucuronide were compared to the parent molecule ($p < 0.05$ by ANOVA analysis) (**Figure 4A**,**B**). Only quercetin, 3′-*O*-methylquercetin, and quercetin-3-*O*-glucuronide offered significant protection against tyrosine chlorination ($p < 0.05$) (**Figure 4A**). Taxifolin exhibited similar activity to quercetin, while luteolin and kaempferol showed lower activity (**Figure 4B**).

Effects on Functional MPO Activity. The effects of quercetin, its metabolites, and structural analogues luteolin, kaempferol, and taxifolin on functional MPO enzyme activity were compared to untreated control neutrophil lysate MPO enzyme activity. The mean rate of guaiacol oxidation by the untreated control neutrophil lysate was 1.36 ± 0.31 units/mL ($n = 3$). Dose-dependent effects on MPO activity were observed for quercetin ($IC_{50} \approx 1.5$ μM) and its metabolites (IC_{50} ranging from 2 to >10 μM) (**Figure 5A**). The four quercetin metabolites as well as the analogues luteolin and kaempferol were less effective at suppressing MPO enzyme activity than quercetin, while the analogue taxifolin had similar activity to quercetin (**Figure 5B**). Quercetin-3′-*O*-sulfate and 3′-*O*-methylquercetin-3-*O*-glucuronide were the only molecules where the decrease in activity as compared to the parent molecule reached significance ($p < 0.05$) (**Figure 5A**).

Effects on Superoxide Production. Superoxide radical ion is a reactive oxygen species produced by the enzyme, NADPH oxidase, in phagocytic cells. Stimulated neutrophils produced approximately 6-fold more superoxide radical ion than unstimulated neutrophils (data not shown). Quercetin ($IC_{50} \approx 4.5$ μM) exhibited dose-dependent inhibition of superoxide formation

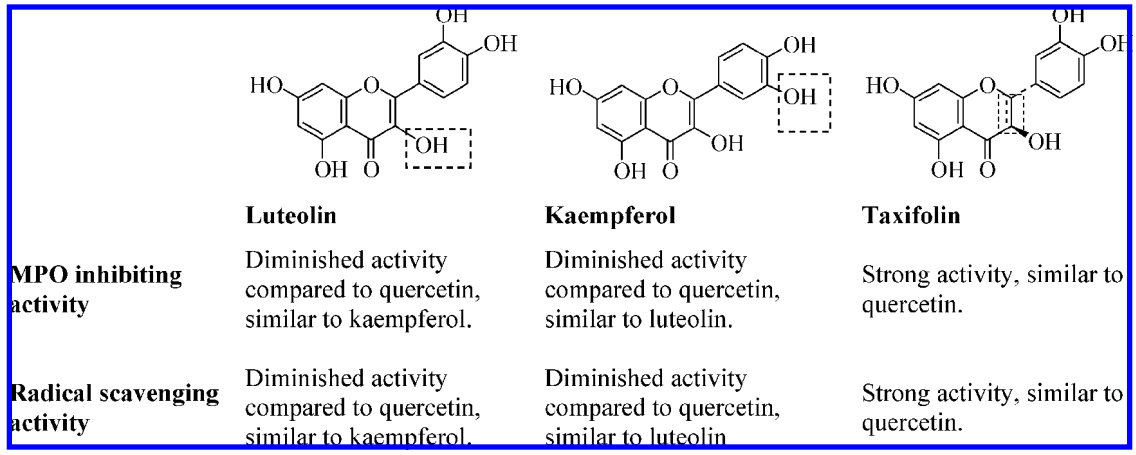

Figure 6. Structural activity relationships for quercetin, luteolin, kaempferol, and taxifolin.

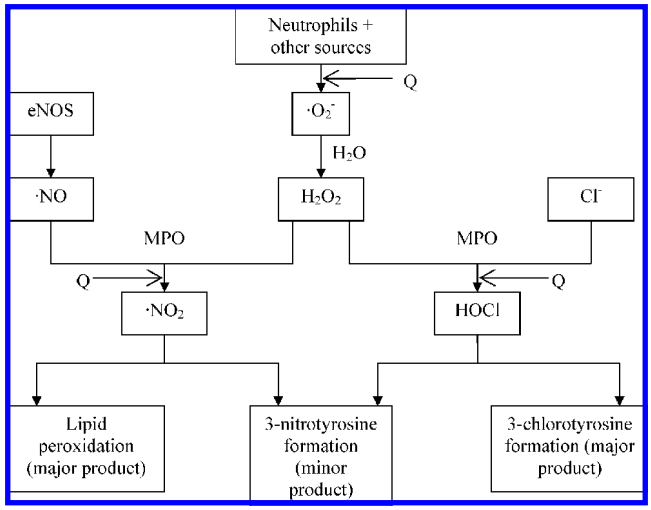

Figure 7. Flow diagram showing possible routes by which MPO can catalyze the modification of LDL. The arrows indicate the points at which quercetin (Q) may inhibit the process.

(data not shown). At 1 μM, quercetin significantly reduced superoxide formation by about 20% (data not shown). Quercetin metabolites showed diminished inhibition, but only quercetin-3′-*O*-sulfate and 3′-*O*-methylquercetin-3-*O*-glucuronide were significantly less active ($p < 0.05$ using ANOVA analysis of the area under dose−response curve) as compared to the parent molecule (data not shown).

DISCUSSION

Our results showed that quercetin and two of its metabolites, 3′-*O*-methylquercetin and quercetin-3-*O*-glucuronide, inhibited neutrophil-mediated modification of LDL as measured by F_2-isoprostanes and 3-chlorotyrosine formation (**Figures 2A** and **4A**) at concentrations that may be achieved in vivo after consumption of a quercetin-rich diet or supplementation. However, the metabolites quercetin-3′-*O*-sulfate and 3′-*O*-methylquercetin-3-*O*-glucuronide consistently showed reduced activity as compared to quercetin. The activities of 3′-*O*-methylquercetin and quercetin-3-*O*-glucuronide were compared with those of luteolin and kaempferol as the absence of the 3-OH group in luteolin and the 3′-OH group in kaempferol are similar to the 3-*O* and 3′-*O* modifications in 3′-*O*-methylquercetin and quercetin-3-*O*-glucuronide. Taxifolin showed similar activity to quercetin, suggesting that removal of the double bond in the C ring had little effect. Taxifolin also showed inhibition of AAPH oxidation of LDL similar to quercetin in contrast to luteolin, kaempferol, and the quercetin metabolites, which were less effective. MPO-catalyzed LDL oxidation was more sensitive to quercetin and its metabolites than the AAPH-initiated LDL oxidation (**Figures 2A** and **3A**). Levels of 3-chlorotyrosine, a product of MPO activity, were reduced in LDL after treatment with all compounds except for quercetin-3′-*O*-sulfate and 3′-*O*-methylquercetin-3-*O*-glucuronide, showing again that these particular metabolic transformations render quercetin less active (**Figure 4A**,**B**). Similar trends in activity were seen when MPO activity was measured in lysed neutrophils (**Figure 5A**,**B**). Metabolic modifications resulting in 3′-*O*-methylquercetin and quercetin-3-*O*-glucuronide did not have a significant effect on MPO inhibitory activity of quercetin. Production of superoxide ion radical by neutrophils was only slightly decreased in the presence of quercetin, 3′-*O*-methylquercetin, and quercetin-3-*O*-glucuronide. These results suggest that inhibition of neutrophil-mediated LDL modification by quercetin and related compounds is predominantly due to inhibition of MPO as well as direct free radical scavenging action as evidenced by the compounds' ability to inhibit AAPH-initiated oxidation.

Quercetin metabolites had diminished ability (IC_{50} values of 2−4 μM) to protect LDL from oxidative damage and to inhibit MPO activity when compared to the parent molecule (IC_{50} value of 1 μM) (**Figures 2A**, **4A**, and **5A**). By comparing the activity of quercetin with its structural analogues (luteolin, kaempferol, and taxifolin), we have proposed a structural activity relationship of quercetin in relation to its free radical scavenging and MPO inhibiting activities (**Figure 6**). Our experiments, which were carried out at physiological concentrations (*24*), showed that the 3′-OH (luteolin) and 3-OH (kaempferol) groups on the B and C rings, respectively, played similar roles in both radical scavenging and MPO inhibiting activities. The 2,3 double bond on the B ring (taxifolin) played a negligible part in protecting LDL against oxidative damage (**Figures 2B**, **3B**, **4B**, and **5B**), because taxifolin retained similar activity to quercetin. Similar observations were reported for cell-free Cu^{2+} and AAPH-mediated LDL oxidation under nonphysiological concentrations (*25, 26*). Our results support the equivalence of the 3′-OH and 3-OH modifications with the observed similarity in activities of 3′-*O*-methylquercetin and quercetin-3-*O*-glucuronide, which were in turn similar to luteolin and kaempferol (**Figures 2A**,**B**, **3B**, **4B**, and **5B**). Interestingly, conjugation at either 3- or 3′-positions did not significantly reduce quercetin activity against MPO-catalyzed events (**Figures 2B**, **4B**, and

5B), unlike that against radical scavenging activities (**Figure 3B**). Our results are consistent with previous reports that the catechol structure at the B ring and the 3-OH group in the C ring account for the radical scavenging effects of flavonoids (*25, 26*). They also agree with a recent report that the presence of a resorcinol group in the A ring is the main contributor to the inhibitory effects of flavonoids on MPO (*27*). The significant lack of activity observed for quercetin-3′-*O*-sulfate may be explained by the possible hydrogen bond between the sulfate group and the 3-OH group (on the C ring) or 4′-OH group (on the B ring), which would limit the availability of the 3-OH group.

We have shown that quercetin protects LDL from neutrophil-mediated modification at physiological concentrations (1 μM). These results were particularly relevant as the neutrophil concentration used reflects their possible numbers at the site of inflammation (*28*). It has been well-established that quercetin with its phenolic OH groups protects against free radical damage via radical scavenging activity (*29*). However, low bioavailability (~1 μM) and metabolic transformation reduce the likely in vivo scavenging action of quercetin. The effect of metabolic transformation on bioactivity has been reported previously (*30, 31*). The importance of quercetin as an antioxidant is brought into question given the higher bioavailability of other in vivo antioxidants, like ascorbic acid and α-tocopherol (*32*), although quercetin may exert synergistic and additive effects with other in vivo antioxidants. Our results suggest that quercetin is not effective at suppressing/scavenging superoxide ion radical production (IC_{50} = 4.5 μM) in activated neutrophils at physiological concentrations. It had been previously reported that quercetin exhibited a dose-dependent inhibition of the generation of superoxide anion radical (IC_{50} = 5.0 μM) from xanthine/xanthine oxidase (*33*). Pincemail et al. (*34*) reported that human MPO activity was inhibited by quercetin (IC_{50} = 3.5 μM) in vitro. We have shown similar inhibition by quercetin on MPO enzyme activity, as well as 3-chlorotyrosine production (an established biomarker of MPO activity) by peripheral neutrophils with an IC_{50} value of 1−1.5 μM (**Figures 4A** and **5A**). Quercetin was also shown to be more effective at protecting against MPO-mediated events (IC_{50} = 1 μM) (**Figures 4A** and **5A**) than superoxide-initiated damage (IC_{50} > 10 μM) in neutrophils. Possible routes by which quercetin may inhibit MPO-catalyzed activities are summarized in **Figure 7**. Flavonoids were reported to be substrates for MPO as they were oxidized by MPO in the absence of LDL to produce reactive free radicals and quinones (*35*). Quercetin was found to be a powerful scavenger of hypochlorous acid (*36*), thus limiting the MPO-catalyzed chlorination of LDL proteins. It was proposed that quercetin also scavenged the $^{\bullet}NO_2$ radical (*37*), which is a powerful oxidant produced by the MPO-catalyzed reaction between hydrogen peroxide and nitrite ions (*38*). Quercetin and other flavonoids have also been shown to act as substrate for chlorination (*39*), thereby protecting the proteins from chlorination damage.

In conclusion, our study has shown that quercetin and at least one of its in vivo major metabolites (quercetin-3-*O*-glucuronide) are potent inhibitors of cell-mediated LDL modification at physiological concentrations, mainly through the inhibition of MPO. The recently reported presence of quercetin glucuronides in human atherosclerotic lesions suggests that quercetin may be available to prevent LDL oxidation in vivo (*40*). These results are particularly interesting because of the potential health benefits of diet derived flavonoids such as quercetin.

ACKNOWLEDGMENT

We thank Dr. Jason Wu for helpful advice.

LITERATURE CITED

(1) Stocker, R.; Keaney, J. F., Jr. Role of oxidative modifications in atherosclerosis. *Physiol. Rev.* **2004**, *84*, 1381–1478.
(2) Fraley, A. E.; Tsimikas, S. Clinical applications of circulating oxidized low-density lipoprotein biomarkers in cardiovascular disease. *Curr. Opin. Lipidol.* **2006**, *17*, 502–509.
(3) Leeuwenburgh, C.; Hardy, M. M.; Hazen, S. L.; Wagner, P.; Ohishi, S.; Steinbrecher, U. P.; Heinecke, J. W. Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J. Biol. Chem.* **1997**, *272*, 1433–1436.
(4) Wang, Z.; Nicholls, S. J.; Rodriguez, E. R.; Kummu, O.; Horkko, S.; Barnard, J.; Reynolds, W. F.; Topol, E. J.; DiDonato, J. A.; Hazen, S. L. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat. Med.* **2007**, *13*, 1176–1184.
(5) Zhang, R.; Shen, Z.; Nauseef, W. M.; Hazen, S. L. Defects in leukocyte-mediated initiation of lipid peroxidation in plasma as studied in myeloperoxidase-deficient subjects: Systematic identification of multiple endogenous diffusible substrates for myeloperoxidase in plasma. *Blood* **2002**, *99*, 1802–1810.
(6) Brennan, M.-L.; Penn, M. S.; Van Lente, F.; Nambi, V.; Shishehbor, M. H.; Aviles, R. J.; Goormastic, M.; Pepoy, M. L.; McErlean, E. S.; Topol, E. J.; Nissen, S. E.; Hazen, S. L. Prognostic value of myeloperoxidase in patients with chest pain. *N. Engl. J. Med.* **2003**, *349*, 1595–1604.
(7) Baldus, S.; Heeschen, C.; Meinertz, T.; Zeiher, A. M.; Eiserich, J. P.; Munzel, T.; Simoons, M. L.; Hamm, C. W. On behalf of the CAPTURE Investigators Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. *Circulation* **2003**, *108*, 1440–1445.
(8) Mocatta, T. J.; Pilbrow, A. P.; Cameron, V. A.; Senthilmohan, R.; Frampton, C. M.; Richards, A. M.; Winterbourn, C. C. Plasma concentrations of myeloperoxidase predict mortality after myocardial infarction. *J. Am. Coll. Cardiol.* **2007**, *49*, 1993–2000.
(9) Arts, I. C.; Hollman, P. C. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr.* **2005**, *81*, 317S–325S
(10) Vita, J. A. Polyphenols and cardiovascular disease: Effects on endothelial and platelet function. *Am. J. Clin. Nutr.* **2005**, *81*, 292S–297S
(11) Waddington, E.; Puddey, I. B.; Croft, K. D. Red wine polyphenolic compounds inhibit atherosclerosis in apolipoprotein E-deficient mice independently of effects on lipid peroxidation. *Am. J. Clin. Nutr.* **2004**, *79*, 54–61.
(12) Kris-Etherton, P. M.; Keen, C. L. Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. *Curr. Opin. Lipidol.* **2002**, *13*, 41–49.
(13) Hodgson, J. M.; Croft, K. D. Dietary flavonoids: Effects on endothelial function and blood pressure. *J. Sci. Food Agric.* **2006**, *86*, 2492–2498.
(14) Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747.
(15) Kroon, P. A.; Clifford, M. N.; Crozier, A.; Day, A. J.; Donovan, J. L.; Manach, C.; Williamson, G. How should we assess the effects of exposure to dietary polyphenols in vitro. *Am. J. Clin. Nutr.* **2004**, *80*, 15–21.
(16) Needs, P. W.; Kroon, P. A. Convenient syntheses of metabolically important quercetin glucuronides and sulfates. *Tetrahedron* **2006**, *62*, 6862–6868.
(17) Wu, J. H. Y.; Ward, N. C.; Indrawan, A. P.; Almeida, C.; Hodgson, J. M.; Proudfoot, J. M.; Puddey, I. B.; Croft, K. D. Effects of α-tocopherol and mixed tocopherol supplementation on markers of oxidative stress and inflammation in type 2 diabetes. *Clin. Chem.* **2007**, *53*, 511–519.
(18) Croft, K. D.; Proudfoot, J. M.; Moulton, C.; Beilin, L. J. The effect of lipoproteins on the release of some eicosanoids by stimulated human leukocytes. A possible role in atherosclerosis. *Eicosanoids* **1991**, *4*, 75–81.

(19) Mori, T. A.; Croft, K. D.; Puddey, I. B.; Beilin, L. J. An improved method for the measurement of urinary and plasma F2-isoprostanes using gas chromatography-mass spectrometry. *Anal. Biochem.* **1999**, *268*, 117–125.
(20) Yamaguchi, Y.; Yoshikawa, N.; Kagota, S.; Nakamura, K.; Haginaka, J.; Kunitomo, M. Elevated circulating levels of markers of oxidative-nitrative stress and inflammation in a genetic rat model of metabolic syndrome. *Nitric Oxide* **2006**, *15*, 380–386.
(21) Klebanoff, S. J.; Waltersdorph, A. M.; Rosen, H. Antimicrobial acitivity of myeloperoxidase. *Methods Enzymol.* **1984**, *105*, 399–403.
(22) Johnston, J.; Richard, B. Measurement of O_2^- secreted by monocytes and macrophages. *Methods Enzymol.* **1984**, *105*, 365–369.
(23) Townend, J. Analysis of variance. In *Practical Statistics for Environmental and Biological Scientists*; J. Wiley & Sons: New Jersey, 2003; pp 107−128.
(24) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Rémésy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–42S
(25) Vaya, J.; Mahmood, S.; Goldblum, A.; Aviram, M.; Volkova, N.; Shaalan, A.; Musa, R.; Tamir, S. Inhibition of LDL oxidation by flavonoids in relation to their structure and calculated enthalpy. *Phytochemistry* **2003**, *62*, 89–99.
(26) Hou, L.; Zhou, B.; Yang, L.; Liu, Z.-L. Inhibition of human low density lipoprotein oxidation by flavonols and their glycosides. *Chem. Phys. Lipids* **2004**, *129*, 209–219.
(27) Meotti, F. C.; Senthilmohan, R.; Harwood, D. T.; Missau, F. C.; Pizzolatti, M. G.; Kettle, A. J. Myricitrin as a substrate and inhibitor of myeloperoxidase: Implications for the pharmacological effects of flavonoids. *Free Radical Biol. Med.* **2008**, *44*, 109–120.
(28) Boos, C. J.; Lip, G. Y. H.; Jilma, B. Endotoxemia, inflammation, and atrial fibrillation. *Am. J. Cardiol.* **2007**, *100*, 986–988.
(29) Santos, M. R.; Mira, L. Protection by flavonoids against the peroxynitrite-mediated oxidation of dihydrorhodamine. *Free Radical Res.* **2004**, *38*, 1011–1018.
(30) Halliwell, B.; Rafter, J.; Jenner, A. Health promotion by flavonoids, tocopherols, tocotrienols and other phenols: Direct or indirect effects? Antioxidant or not? *Am. J. Clin. Nutr.* **2005**, *81* (Suppl.), 268S–276S
(31) Lotito, S. B.; Frei, B. Dietary flavonoids attenuate tumor necrosis factor α-induced adhesion molecule expression in human aortic endothelial cells: structure-function relationships and activity after first pass metabolism. *J. Biol. Chem.* **2006**, *281*, 37102–37110.
(32) Lesser, S.; Wolffram, S. Oral bioavailability of the flavonol quercetin—A review. *Curr. Top. Nutraceut. Res.* **2006**, *4*, 239–256.
(33) Selloum, L.; Reichl, S.; Muller, M.; Sebihi, L.; Arnhold, J. Effects of flavonols on the generation of superoxide anion radicals by xanthine oxidase and stimulated neutrophils. *Arch. Biochem. Biophys.* **2001**, *395*, 49–56.
(34) Pincemail, J.; Deby, C.; Thirion, A.; de Bruyn-Dister, M.; Goutier, R. Human myeloperoxidase activity is inhibited in vitro by quercetin. Comparison with three related compounds. *Cell. Mol. Life Sci.* **1988**, *44*, 450–453.
(35) Kostyuk, V. A.; Kraemer, T.; Sies, H.; Schewe, T. Myeloperoxidase/nitrite-mediated lipid peroxidation of low-density lipoprotein as modulated by flavonoids. *FEBS Lett.* **2003**, *537*, 146–150.
(36) Selloum, L.; Djelili, H.; Sebihi, L.; Arnhold, J. Scavenger effect of flavonols on HOCl-induced luminol chemiluminescence. *Luminescence* **2004**, *19*, 199–204.
(37) Schewe, T.; Sies, H. Myeloperoxidase-induced lipid peroxidation of LDL in the presence of nitrite. Protection by cocoa flavanols. *Biofactors* **2005**, *24*, 49–58.
(38) Brennan, M.-L.; Wu, W.; Fu, X.; Shen, Z.; Song, W.; Frost, H.; Vadseth, C.; Narine, L.; Lenkiewicz, E.; Borchers, M. T.; Lusis, A. J.; Lee, J. J.; Lee, N. A.; Abu-Soud, H. M.; Ischiropoulos, H.; Hazen, S. L. A tale of two controversies. Defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J. Biol. Chem.* **2002**, *277*, 17415–17427.
(39) Binsack, R.; Boersma, B. J.; Patel, R. P.; Kirk, M.; White, C. R.; Darley-Usmar, V.; Barnes, S.; Zhou, F.; Parks, D. A. Enhanced antioxidant activity after chlorination of quercetin by hypochlorous acid. *Alcohol: Clin. Exp. Res.* **2001**, *25*, 434–443.
(40) Kawai, Y.; Nishikawa, T.; Shiba, Y.; Saito, S.; Murota, K. Macrophage as a target of quercetin glucuronides in human atherosclerotic arteries: Implication in the anti-atherosclerotic mechanism of dietary flavonoids. *J. Biol. Chem.* **2008**, *283*, 9424–9434.

Received for review January 30, 2008. Revised manuscript received March 12, 2008. Accepted March 14, 2008. This work was supported by grants from the National Heart Foundation of Australia, National Health and Medical Research Council (Australia), and Biotechnology and Biological Sciences Research Council (United Kingdom). W.M.L. thanks the University of Western Australia for an International Research Fees Scholarship.

JF8003042