Quercetin Protects Against Linoleic Acid-Induced Porcine Endothelial Cell Dysfunction

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ABSTRACT Consumption of plant phenolics, such as quercetin, may be associated with decreased risk of cardiovascular disease by stabilizing and protecting vascular endothelial cells against oxidative and proinflammatory insults. The present study focused on the effect of quercetin on linoleic acid–induced oxidative stress and the inflammatory pathways of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). Because the transcription factor peroxisome proliferator activated receptor γ (PPARγ) was reported to downregulate inflammatory pathways, we further investigated the effect of quercetin on PPARγ. Porcine pulmonary-arterial endothelial cells were activated with linoleic acid in the presence or absence of quercetin. Oxidative stress was markedly induced by endothelial cell exposure to linoleic acid and diminished by treatment with quercetin as measured via the oxidation of 2',7'-dichlorofluorescin. Quercetin reduced linoleic acid–mediated binding activity of NF-κB and AP-1 and mRNA levels of inflammatory genes such as interleukin-6 (IL-6) and vascular cell adhesion molecule-1 (VCAM-1). Cotreatment of linoleic acid plus quercetin or vitamin E also decreased linoleic acid–induced binding activity of PPARγ. These data suggest that quercetin has potent antioxidative and anti-inflammatory properties and protects endothelial cells against linoleic acid–mediated cell dysfunction. J. Nutr. 134: 771–775, 2004.

KEY WORDS: • atherosclerosis • fatty acids • quercetin • PPAR

Atherosclerotic lesions are thought to be initiated by vascular endothelial cell dysfunction. A damaged endothelium is less effective as a selectively permeable barrier to plasma components (1,2). The endothelium interacts with the blood and underlying tissues, serves as both a pro- and antithrombotic surface, and releases regulatory factors important in modulating vascular tone. Factors implicated in the pathogenesis of atherosclerosis include chronic and cumulative metabolic alterations of the endothelium induced by numerous activating molecules, such as certain lipids, prooxidants, and inflammatory cytokines. These risk factors may contribute to an overall cellular imbalance of the oxidative stress/antioxidant balance, thus leading to chronic activation of the endothelium and alterations of the endothelial barrier function, which can result in accelerated uptake of cholesterol-rich lipoproteins into the vessel wall.

After consumption of high-energy foods, triglyceride-rich lipoproteins are elevated, and hydrolysis of triglycerides by lipoprotein lipase occurs in proximity to the endothelial surface (3). An excessive local concentration of fatty acid anions may cause endothelial injury and therefore initiate the onset of atherosclerosis. PUFA are more susceptible to lipid peroxidation than SFA, in particular when insufficiently protected by antioxidants. Thus, if oxidative stress is a critical underlying parameter of atherosclerosis (4), then high serum PUFA concentrations may indicate a higher risk of atherosclerosis (5).

Antioxidants, such as vitamin E, can significantly reduce the linoleic acid–mediated endothelial cell activation and loss of endothelial integrity (6). Quercetin, like other polyphenolics, possesses high antioxidant abilities to inhibit free radical processes in cells (7), including the prevention of lipid peroxidation (8).

Numerous oxidative stress-sensitive transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (9) can mediate an inflammatory response due to oxidative stress by inducing gene transcription of adhesion molecules and cytokines such as vascular cell adhesion molecule-1 (VCAM-1) and interleukin-6 (IL-6). Antioxidants such as quercetin could protect plasma lipids from oxidation, thereby preventing the induction of inflammatory events. Thus, quercetin could help maintain the integrity of the endothelium.

There is increasing evidence that peroxisome proliferator activated receptors (PPARs) can modulate inflammatory events and are thus antiatherogenic (10). For example, PPARγ can interfere negatively with NF-κB, signal transducer...
and activator of transcription (STAT), and AP-1 signaling pathways (11,12). This could be by preventing transcription factors from binding to their target sequences (12,13), possibly through an interaction with a subunit of the transcription factors (e.g., p65) (12). Because most of the proinflammatory genes are under the control of the AP-1 and NF-κB signaling pathways, and because PPARs can counterregulate a wide spectrum of proinflammatory genes, anti-inflammatory compounds may act through PPAR signaling. The binding pockets of PPARs are open to a variety of naturally occurring lipid-like substances acting as low-affinity ligands (14). Because quercetin is a lipophilic, polyphenolic substance with a chemical structure that could potentially fit the binding pocket of PPARs, we investigated whether the anti-inflammatory properties of quercetin could be through the activation of PPARγ.

In this study we aimed to demonstrate that quercetin has endothelium-protective effects by preventing linoleic acid-induced oxidative stress formation and by decreasing the activation of oxidant-sensitive pathways.

MATERIALS AND METHODS

Cell culture and experimental media. Endothelial cells were isolated from porcine pulmonary arteries as described previously (15). Tissues obtained during routine slaughters were donated by the College of Agriculture, University of Kentucky. Cells were subcultured in medium 199 (M-199) containing 10% (v:v) fetal bovine serum (FBS, HyClone Laboratories) using standard techniques.

The experimental media were composed of M-199 enriched with 5% (v:v) FBS and fatty acids (90 μmol/L). Fatty acids (≥99% pure) were obtained from Nu-Chek-Prep. Preparations of experimental media with fatty acids were made as described previously (16). Thus, fatty acids were introduced into the media bound to serum albumin. Quercetin (10–50 μmol/L) and vitamin E (25 μmol/L) were added from stock solutions in dimethylsulfoxide (DMSO) and ethanol, respectively. Controls and fatty acid groups not treated with quercetin or vitamin E contained an equal amount of DMSO or ethanol. The final DMSO concentration in the media never exceeded 0.05% (v/v) in all treatment groups. For most experimental settings, cells were treated with quercetin and fatty acids for 6 h. Vitamin E was added 18 h before fatty acid treatment.

Measurement of oxidative stress. Cellular oxidation was determined by 2′,7′-dichlorofluorescein (DCF) fluorescence as described by Mattson et al. (17). This measurement of cell oxidation utilizes an imaging technique based on the conversion of 2′,7′-dichlorofluorescin into fluorescent 2′,7′-dichlorofluorescein as a result of activation with reactive oxygen species (ROS), primarily peroxyl radicals and peroxides. After treatment of endothelial cells with linoleic acid for 6 h, cells were loaded with 100 μmol/L 2,7-dichlorofluorescin diacetate (Molecular Probes) by incubation for 30 min. Before analysis for oxidative stress, cells were washed 3 times in HEPES buffer. In experiments utilizing H2O2 as an inducer of oxidative stress, 0.1 mmol/L H2O2 for up to 60 min (Fig. 1B). Quercetin at 25 and 50 μmol/L significantly reduced the generation of free radicals. Increasing the concentration of quercetin to 50 μmol/L quercetin for 6 h. Significantly different from 25 and 50 μmol/L quercetin for 6 h. Significant effects were observed at 25 and 50 μmol/L quercetin (Fig. 1A).

Quercetin attenuates linoleic acid and H2O2-induced oxidative stress. Quercetin dose-dependently reduced oxidative stress when cells were cotreated with 90 μmol/L linoleic acid and 10, 25, or 50 μmol/L quercetin for 6 h. Significant effects were observed at 25 and 50 μmol/L quercetin (Fig. 1A). Similar effects were obtained in endothelial cells exposed to 0.1 mmol/L H2O2 for up to 60 min (Fig. 1B). Quercetin at 25 and 50 μmol/L significantly reduced the generation of free radicals. Increasing the concentration of quercetin to 50 μmol/L quercetin for 6 h.

RESULTS

Quercetin attenuates linoleic acid and H2O2-induced oxidative stress. Quercetin dose-dependently reduced oxidative stress when cells were cotreated with 90 μmol/L linoleic acid and 10, 25, or 50 μmol/L quercetin for 6 h. Significant effects were observed at 25 and 50 μmol/L quercetin (Fig. 1A). Similar effects were obtained in endothelial cells exposed to 0.1 mmol/L H2O2 for up to 60 min (Fig. 1B).

Statistical analysis. The data were quantified and analyzed using the Scion Image and Sigma Stat software, respectively. Comparisons between treatments were made by 1- or 2-way ANOVA with post-hoc comparisons of the means made by Tukey’s tests. A statistical probability of P < 0.05 was considered significant.

FIGURE 1 Fatty acid- (A) and H2O2- (B) induced oxidative stress in primary endothelial cells. Cells were grown in 24-well plates, exposed to 90 μmol/L fatty acid and/or various concentrations of quercetin for 6 h. Oxidative stress was determined spectrometrically by measuring the conversion of dichlorofluorescin to dichlorofluorescein. Q, quercetin; L, linoleic acid; H2O2; concentrations in μmol/L. Values are means ± SEM, n = 3. Different from the control, P < 0.05; #linoleic acid + quercetin (L + Q) and H2O2 + quercetin (H + Q) different from linoleic acid (L), P < 0.05.
μmol/L did not increase protection against free radical formation compared with 25 μmol/L quercetin.

Quercetin decreases linoleic acid–induced binding activity of NF-κB and AP-1. Linoleic acid increased the DNA binding activity of both transcription factors NF-κB and AP-1 as determined by EMSA. Cotreatment of quercetin and linoleic acid for 6 h downregulated the activation of NF-κB and AP-1 (Figs. 2A and B, respectively). Consistent with the observations made in measuring oxidative stress, 25 μmol/L appeared to be the most effective concentration of quercetin with no additional benefit of higher concentrations to downregulate NF-κB (Fig. 2A). Maximal downregulation of linoleic acid–induced AP-1 binding activity already occurred at 10 μmol/L quercetin (Fig. 2B).

Quercetin protects against linoleic acid–induced IL-6 and VCAM-1 gene expression. VCAM-1 expression was upregulated after a 6-h exposure to linoleic acid (Fig. 3A), and coexposure to quercetin protected against this effect. The quercetin-induced decrease in VCAM-1 gene expression was concentration dependent, with complete blockage of linoleic acid–induced gene expression in the presence of 25 μmol/L quercetin (Fig. 3A).

Cytokine IL-6 expression was upregulated in response to linoleic acid treatment (Fig. 3B). Quercetin was less potent in downregulating IL-6 mRNA compared with the effects seen in the downregulation of NF-κB and AP-1 binding activity or VCAM-1 mRNA. Indeed, quercetin was effective only when applied at higher concentrations (50 μmol/L).

Quercetin and vitamin E decrease linoleic acid–induced PPARγ binding activity. PPARγ protects cells against proinflammatory and prooxidative insults, and linoleic acid is a natural ligand for this transcription factor. Therefore, the effects of linoleic acid and/or quercetin on PPARγ DNA binding activity were assessed in the present study. Exposure to linoleic acid for 6 h markedly induced PPARγ activity (Figs. 4A and B). Although quercetin alone did not affect binding of this transcription factor, it diminished PPARγ activation in cells cotreated with linoleic acid. To assess whether the effects of quercetin on activation of PPARγ were specific, cells were pretreated with another antioxidant, vitamin E (25 μmol/L for 18 h) and treated with linoleic acid for 6 h. Similar to the effects exerted by quercetin, vitamin E also protected against linoleic acid–induced binding activity of PPARγ (Fig. 4B).
Effects of linoleic acid, quercetin (A) and vitamin E (B) on PPARγ activity in endothelial cells. Primary endothelial cells were cotreated with 90 μmol/L linoleic acid and 10, 25, or 50 μmol/L quercetin for 6 h or pretreated with 25 μmol/L vitamin E 18 h before fatty acid treatment. The binding activity of the transcription factor was determined by EMSA. Q, quercetin; L, linoleic acid; concentrations in μmol/L. Values are means ± SEM, n = 3. *Different from the control, P < 0.05; #linoleic acid + quercetin (L + Q) different from linoleic acid (L), P < 0.05.

DISCUSSION

Foods rich in plant phenolics may be associated with a decreased risk of atherosclerosis by protecting vascular endothelial cells against proinflammatory lipids. In the present study, we demonstrated an overall antioxidant and anti-inflammatory effect of quercetin. We reported earlier (20) that linoleic acid markedly increases oxidative stress in cultured endothelial cells as measured by DCF fluorescence. Here we show that cotreatment with quercetin significantly inhibits the formation of ROS after treating endothelial cells with linoleic acid. The reduction in the generation of ROS is likely due to a direct scavenging effect of quercetin. The chemical structure of quercetin allows the prediction of antioxidant properties of this molecule. It has been suggested that quercetin becomes oxidized to a semiquinone and quinine (21) while neutralizing ROS. Both the semiquinone and the quinine could be regenerated by glutathione and vitamins E and C (21). Therefore, quercetin could be a valuable contributor to the cellular oxygen defense system. On the other hand, when applied in high concentrations and for long incubation times, quercetin was shown to exhibit prooxidant effects, as reported by others (21,22); we observed this also upon extended treatment with high levels of this phenolic compound (data not shown). As a consequence, the antioxidant capacities of quercetin have been challenged. However, a prooxidant effect of quercetin in vivo seems to be unlikely because plasma quercetin levels do not reach concentrations that induce oxidative stress in in vitro experiments (23).

The antioxidant properties of quercetin also could be responsible in part for the anti-inflammatory effects observed in the present study. We showed that quercetin can downregulate the linoleic acid–induced activation of both NF-κB and AP-1. These are oxidative stress–sensitive transcription factors that can therefore be modified by oxidants and antioxidants. The precise way in which linoleic acid induces an inflammatory response in endothelial cells is not clear. However, oxidation products of linoleic acid might be directly involved in the activation of NF-κB. Lipid peroxides rather than H2O2 were suggested to mediate the activation of NF-κB in response to other lipid stress (24). It is possible that lipid metabolites and derivatives, including oxidized fatty acids, can induce the inflammation, in addition to the native linoleic acid itself. Furthermore, quercetin was suggested to suppress inhibitory IκB kinase (25) and c-Jun kinase (26), which subsequently could lead to suppression of NF-κB and AP-1 activation. Activation of NF-κB leads to expression of inflammatory cytokines and adhesion molecules, resulting in recruitment of monocytes and accelerated development of atherosclerosis (27). Because most of the proinflammatory genes are under the control of the AP-1 and NF-κB signaling pathways (28) and quercetin can counterregulate these pathways, the potent anti-inflammatory properties of quercetin are clearly demonstrated. In support of its anti-inflammatory properties, we showed that quercetin can block the linoleic acid–induced expression of both VCAM-1 and IL-6, a downstream event of NF-κB activation (29).

Because PPARγ can interfere negatively with NF-κB, STAT, and AP-1 signaling pathways (11,12), we further investigated whether quercetin can affect PPARγ binding activity. Even though both PPARα and PPARγ were reported to be anti-inflammatory and antiatherogenic, PPARs is involved in lipid metabolism and could therefore be activated independently of fatty acid modification by oxidation. Thus, the present study focuses on PPARγ. Although quercetin has a chemical structure that could potentially be a ligand for PPARγ, exposure to quercetin alone did not activate this transcription factor. Consistent with the known overall effects of fatty acids on PPARγ activation, treatment with linoleic acid induced PPARγ binding activity in endothelial cells. However, cotreatment with quercetin markedly downregulated linoleic acid–induced PPARγ activation. These results are consistent with earlier reports showing that quercetin can mediate downregulation of PPARγ in transiently transfected macrophages (30) and in murine epidermal keratinocytes (31). Because linoleic acid can induce oxidative stress and inflammation and at the same time activate PPAR, we suspected that lipid oxidation is involved in activating the PPAR pathway. To further address the question whether the decreased PPARγ activation observed in the present study is specific for quercetin, endothelial cells were treated with another antioxidant (vitamin E) followed by exposure to linoleic acid. Vitamin E also prevented the linoleic acid–mediated activation of PPARγ. These data suggest that quercetin can reduce the linoleic acid–mediated activation of PPARγ due to its anti-oxidant effect and the prevention of lipid oxidation. Previous studies indicated that oxidized products of linoleic acid are good ligands for PPARs (32). Quercetin may act as an anti-
oxidan; thus the decreased formation of oxidized products of linoleic acid might be a reason for the lower binding of linoleic acid to PPARγ in the presence of quercetin.

These data suggest that the quercetin-mediated protection observed against linoleic acid–induced endothelial cell activation is independent of PPARγ signaling. In addition, it appears that the diminished PPARγ DNA binding activity observed in cells exposed to linoleic acid plus quercetin may be related to a general antioxidant effect of this polyphenolic. Indeed, PPARs may act as critical rescue molecules by down-regulating oxidative stress–sensitive and inflammatory signaling pathways. In cells that are protected by antioxidants such as quercetin and vitamin E, oxidative stress–sensitive pathways, including PPARγ, are less likely to become activated.

Overall, our data suggest that quercetin is a potent antioxidant and anti-inflammatory substance, which can protect the endothelium against oxidative stress and inflammatory events, despite downregulation of PPARγ activation. Specifically, quercetin inhibited linoleic acid–induced activation of oxidative stress–sensitive pathways, such as NF-κB and AP-1, and inflammatory genes, such as VCAM-1 and IL-6. These data support the hypothesis that plant phenolics such as quercetin can help prevent the development of atherosclerosis by down-regulating the expression of inflammatory cytokines and adhesion molecules.

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