Quercetin Glucuronide Inhibits Cell Migration and Proliferation by Platelet-Derived Growth Factor in Vascular Smooth Muscle Cells

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Abstract. Many epidemiologic studies have reported that dietary flavonoids provide protection against cardiovascular disease. Quercetin, a member of the bioflavonoids family, has been proposed to have anti-inflammatory, anti-atherogenic, and anti-hypertensive properties leading to the beneficial effects against cardiovascular diseases. Recent studies demonstrated that orally administered quercetin appeared in plasma as glucuronide-conjugated forms in rats and humans. Therefore, we examined the effect of chemically synthesized quercetin glucuronide on platelet-derived growth factor (PDGF)-induced cell migration and kinase activation in cultured rat aortic smooth muscle cells (RASMCs). PDGF-induced RASMC migration was inhibited by quercetin 3-O-β-D-glucuronide (Q3GA). Q3GA also attenuated PDGF-induced cell proliferation in RASMCs. PDGF activated extracellular-signal regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein (MAP) kinase, and Akt in RASMCs. PDGF-induced JNK and Akt activations were suppressed by Q3GA, whereas ERK1/2 and p38 MAP kinase activations were not affected. We also confirmed that PDGF-induced JNK and Akt activations were inhibited by antioxidants, N-acetylcysteine and diphenyleneiodonium chloride, in RASMCs. These findings suggest Q3GA would be an active metabolite of quercetin in plasma and may possess preventing effects for cardiovascular diseases relevant to vascular smooth muscle cell disorders.

Keywords: quercetin glucuronide, platelet-derived growth factor, migration, proliferation, vascular smooth muscle cell

Introduction

Quercetin (3,3',4',5,7-pentahydroxyflavone), a member of the bioflavonoids family, is one of the most widely dietary polyphenolic compounds in foods including vegetables, fruits, tea, and wine (1). Quercetin has been shown to possess anti-atherogenic, anti-inflammatory, anti-coagulative, and anti-hypertensive properties (1 – 3). In addition, within the bioflavonoid family, quercetin is the most potent scavenger of reactive oxygen species (ROS) (4). The antioxidant activity of quercetin has been shown to contribute to its protective effects against the cardiovascular diseases (5). In our previous study, it was reported that quercetin inhibited Shc- and phosphatidylinositol 3-kinase (PI3-kinase)-mediated c-Jun N-terminal kinase (JNK) activation by angiotensin II in rat aortic smooth muscle cells (RASMCs) (6). Several studies provided that quercetin from the diet is partly absorbed into the body and accumulated in the circulation (7, 8). However, it was recently demonstrated that quercetin 3-O-β-D-glucuronide (Q3GA) and quercetin-3′-sulfate are the major quercetin conjugates in human plasma, in which aglycone could not be detected (9 – 11). It was also shown that orally administered quercetin is converted to its conjugates before accumulation in rat plasma (12, 13). We have already reported that Q3GA inhibits Ang II–induced vascular smooth muscle cell (VSMC) hypertrophy via its inhibitory effect on JNK. 

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and the AP-1 signaling pathway (14). By using the electron paramagnetic resonance (EPR) method, we also demonstrated that both Q3GA and quercetin scavenged 1,1-diphenyl-2-pirclylhydrazyl (DPPH) radical (14). Our above-described findings suggested that Q3GA resembles quercetin with respect to its antioxidative potency. Interestingly, orally administered quercetin appeared in plasma as glucuronide-conjugated forms in rats and humans, although the quercetin aglycone form could not be detected (10, 11). Thus, the pharmacological function of dietary quercetin should be exerted exclusively by its conjugated metabolites. Therefore in the present study, we examined the effects of the chemically synthesized quercetin glucuronide, Q3GA, on platelet-derived growth factor (PDGF)-induced mitogen-activated protein (MAP) kinases and PI3K activation in VSMCs.

The primary cause of many fatal cardiovascular diseases is believed to be atherosclerosis (15). During atherogenesis and the progression of the disease, chronic inflammatory responses induce vascular wall remodeling or the generation of neointima and thickening of the tunica media, which lead to the development of plaque and artery stenosis (16). The neointima and thickened media are primarily composed of abnormally proliferating and migrating VSMCs (17 – 20). VSMC migration is induced by various inflammatory cytokines and chemokines. PDGF has been recognized as a major mitogen and one of the most important growth factors, and it also stimulates VSMC migration (21, 22). It has been reported that PDGF-induced VSMC migration and proliferation mediated by MAP kinases, PI3-kinase /Akt, and many other kinases (23 – 25).

In the present study, we examined the effect of chemically synthesized Q3GA, as an in vivo form, on PDGF-induced cell migration and proliferation in VSMCs. Although most of the in vitro pharmacological studies have been carried out using only the quercetin aglycone form, experiments using Q3GA would be important to discover the in vivo mechanisms through which quercetin exerts its protective effect against cardiovascular diseases.

Materials and Methods

Cells and materials

RASMCs were isolated from male Sprague-Dawley rats weighing 200 – 250 g and maintained in 10% fetal bovine serum and Dulbecco’s modified Eagle’s medium (DMEM) as described previously (26). Cells were seeded (1 – 2 × 10⁴ cells/cm²) and grown to subconfluence in 60-mm culture dishes (IWAKI, Osaka) in DMEM – 10% FBS. For the experiments, cells from passages 3 – 8 were used after 24 – 48 h of serum depletion. PDGF-BB (human, recombinant), quercetin, N-acetylcysteine (NAC), and diphenyleleniodonium chloride (DPI) were purchased from Sigma (St. Louis, MO, USA). Anti-phospho-extracellular-signal regulated kinase (ERK)1/2 (Thr202/Tyr204) antibody, anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibody, anti-p38 MAP kinase antibody, anti-phospho-Akt (Ser473) antibody, Akt antibody, and the JNK activity assay kit were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-ERK1/2 antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). SP600125, a JNK inhibitor, was from BioMol Research Laboratories Inc. (Plymouth Meeting, PA, USA). LY294002, a PI3-kinase inhibitor, was purchased from Calbiochem (San Diego, CA, USA). All other chemicals were of reagent grade, obtained from commercial sources, and used without further purification.

Chemical synthesis of quercetin glucuronide

Q3GA was chemically synthesized using the Koenigs-Knorr reaction as described previously (14). In brief, quercetin (1 g) and acetobromo-α-D-glucuronic acid methyl ester (5 g) were dissolved in 50 ml of N,N-dimethylformamide. After the addition of potassium carbonate (1 g), the mixture was stirred for 2 h at room temperature. The resulting mixture was added to 300 g of cooled water and then adjusted to an acidic condition by adding a few drops of formic acid. The precipitate in the acidic solution was separated by centrifugation (10,000 × g, 15 min, 4°C). The precipitate was further washed with 50 ml of 0.2% formic acid and then dissolved in 50 ml of chloroform and the same volume of methanol. After the removal of chloroform and methanol by evaporation, the residue was subjected to freeze-drying to eliminate residual water. Twenty-eight percent sodium methylate (1.5 ml) in methanol was added to the freeze-dried material dissolved in 50 ml of chloroform/dry methanol (1:1, v/v), and then the material was dissolved in 50 ml of dry methanol. Then the mixture was kept at 4°C for 30 min to remove acetyl moieties from the tetraacetylglucose bound to quercetin. Fifty milliliters of water was added and the methyl ester was hydrolyzed by letting the mixture stand for 30 min at room temperature. The solution was neutralized with 3.7 ml of 2.0 N hydrochloric acid and then it was adjusted to an acidic condition by adding a few drops of formic acid. The acidic solution was filtered through No. 2 filter paper (Advantec, Tokyo). The filtrate was evaporated and again dissolved in 30 ml of water and chromatographed on a column (4.0 × 40 cm) of Toyopearl HW 40 (Tosoh, Tokyo). Products were eluted with a linear gradient from methanol/water/formic acid (20:79.9:0.1, v/v/v, 500 ml) to methanol (500 ml).
The elution of the products was monitored by the absorbance at 360 nm of each fraction (15 ml/fr.). The quercetin glucuronide fractions were further purified by preparative HPLC (Tosoh CCPE II system, 360 nm) on an ODS column (20 × 250 mm, 10 μm, Inertsil ODS-3; GL Sciences, Inc., Tokyo) with a linear gradient elution from methanol/water/formic acid (20:79:9:0.1, v/v/v) to methanol. The Q3GA fraction was identified with FABMS (fast atom bombardment mass spectrometry) and NMR analyses as described previously (14) and collected for the experiments.

Migration assay

To assess cell migration, a modified Boyden chamber assay was performed by minor modification to the method described previously (27). The assay was performed using Transwell chambers (6.5 mm, model 3422; Costar, Cambridge, MA, USA) with an 8-μm pore polycarbonate membrane. The underside of the polycarbonate membrane was coated with 5 μg/ml collagen I rat tail (3.78 mg/ml, model 35-4236; Collaborative Biochemical Products, Bedford, MA, USA) overnight at 4°C. Then, the lower chamber was blocked with DMEM/0.1% bovine serum albumin for 30 min at room temperature. Growth-arrested cells were harvested, washed, and suspended in serum-free DMEM. Cells were added to the upper chamber of the Transwell at 5 × 10^5 cells in 100 μl/well. A total of 600 μl of serum-free DMEM was added to the lower chamber and then pretreated with or without Q3GA, SP600125, or LY294002 in the lower chamber for 30 min at 37°C followed by treatment with or without PDGF in the lower chamber, and cells were allowed to migrate at 37°C. After 6 h of incubation, the nonmigratory cells were removed from the upper surface of the membrane by scraping them off with cotton swabs. The membrane was fixed with methanol, stained with Diff-Quik antibody (horseradish peroxidase–conjugated). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and were quantified by densitometry in the linear range of film exposure using an Epson Perfection 2580 Photo Scanner (Epson America, Inc., Long Beach, CA, USA) and imageJ 1.37v software (http://rsb.info.nih.gov/ij/).

Proliferation assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay was used for estimating RASMC proliferation as described previously (28). Briefly, when RASMCs reached 4%–50% confluence in 35-mm cell culture dishes (IWAKI), growth was arrested using DMEM without FBS for 24–48 h. Q3GA, SP600125, or LY294002 were added and incubated for 30 min prior to PDGF stimulation for 48 h. MTT was added at a final concentration of 0.5 mg/ml, and after further 2-h incubation, RASMCs were lysed with isopropanol containing 0.04 M HCl. MTT reduction was read at 550 nm by a spectrophotometer.

Preparation of cell lysate for immunoblotting and JNK activity assay

Quiescent control cells were treated with or without PDGF and Q3GA at the indicated concentrations for the indicated periods of time. Then, incubation media were discarded, and the cells were lysed with cell lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). After freeze-thawing, lysed cells were transferred to microcentrifuge tubes, sonicated (Handy Sonic UR-20 P; Tomy Seiko Co., Ltd., Tokyo) on ice, and centrifuged at 20,000 × g for 20 min at 4°C. The protein concentrations of the supernatants were measured with a protein assay kit (BIO-RAD, Hercules, CA, USA) and stored at −80°C until performing immunoblotting and the JNK activity assay.

Western blotting analysis

ERK1/2, p38 MAP kinase, and Akt activities in RASMCs were measured by western blot analysis as described previously (26). Cell lysates were subjected to sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose membranes (Hybond™-ECL; Amersham Pharmacia Biotech, Buckinghamshire, England). The membranes were blocked for 1 h at room temperature with 5% bovine serum albumin. The blots were then incubated for 12 h with anti-phospho-ERK1/2, anti-phospho-p38 MAP kinase, and anti-phospho-Akt antibodies followed by incubation for 1 h with a secondary antibody (horseradish peroxidase–conjugated). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and were quantified by densitometry in the linear range of film exposure using an Epson Perfection 2580 Photo Scanner (Epson America, Inc., Long Beach, CA, USA) and imageJ 1.37v software (http://rsb.info.nih.gov/ij/).

JNK activity assay

JNK activity was measured with a commercially available kit based on phosphorylation of recombinant c-Jun (Cell Signaling Technology, Inc.). After treatment, cells were rinsed twice with ice-cold PBS, scraped off the plates into lysis buffer (included in the kit), and sonicated three times on ice. After removing the cell
debris by centrifugation (16,000 × g, 20 min, 4°C), the protein content in the supernatant was measured by a protein assay kit (Bio-Rad). Equal amounts of protein (300 μg) were then immunoprecipitated with c-Jun (1-89) fusion protein beads overnight. After washing the beads, kinase assays were performed according to the instructions of the manufacturer. The beads were loaded on a 10% SDS–polyacrylamide gel, and immunoblotting was performed with an antibody against phospho-specific c-Jun (29).

**Statistical analyses**

Values are each the mean ± S.D. from experiments performed on five different occasions. Two-way ANOVA was used to determine the significance among groups, after which a modified t-test with Bonferroni’s post hoc test was used for comparisons between individual groups. A value of *P*<0.05 was considered to be statistically significant.

**Results**

**The effects of Q3GA on PDGF-induced cell migration and proliferation in RASMCs**

At first, we confirmed that PDGF increased RASMC migration in a concentration-dependent manner (0.1, 1, 5, and 10 ng/ml) by a modified boyden chamber assay as described in the Materials and Methods section (data not shown). To examine whether Q3GA affects PDGF-induced RASMC migration, growth-arrested RASMCs were treated with or without Q3GA (1, 10, and 100 μM) 30 min prior to stimulation with PDGF (10 ng/ml, 6 h). As shown in Fig. 1, Q3GA pretreatment significantly suppressed PDGF-induced RASMC migration in a concentration dependent manner (10 and 100 μM). PDGF (10 ng/ml) stimulation time-dependently caused RASMC proliferation (24 and 48 h) (data not shown). As shown in Fig. 2, Q3GA significantly attenuated PDGF (10 ng/ml, 48 h)-induced RASMC proliferation (10 and 100 μM).

**The effects of Q3GA on PDGF-induced ERK1/2, p38 MAP kinase, JNK, and Akt activations**

To evaluate the relative magnitude of ERK1/2, p38 MAP kinase, JNK, and Akt (a downstream target of PI3-kinase) activations by PDGF, RASMCs were exposed to PDGF (10 ng/ml) for the indicated periods of time. PDGF rapidly activated ERK1/2 and p38 MAP kinase (peak at 5 min) and JNK (peak at 10 min), then gradually declined (Fig. 3A). Akt was activated within 10 min by PDGF stimulation and the activation was sustained until 60 min (Fig. 3A). PDGF concentration-dependently increased the activation of each of these...
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kinases as measured at each peak time of activation (Fig. 3B). To examine the effects of Q3GA on the activations of these kinases in RASMCs, the cells were pretreated with Q3GA for 30 min before the addition of PDGF (10 ng/ml) for 5 min for ERK1/2 or p38 MAP kinase activations and for 10 min for JNK or Akt activations. As shown in Fig. 4, exposure to Q3GA alone had no effect on the activations of ERK1/2, p38 MAP kinase, JNK, and Akt in RASMCs. PDGF-induced JNK and Akt activations were inhibited by Q3GA in a concentration-dependent manner (10 and 100 μM) (Fig. 4). In contrast, ERK1/2 and p38 MAP kinase activations were not influenced by Q3GA (Fig. 4). These results suggested that JNK and Akt, but not ERK1/2 and p38 MAP kinases, were specifically sensitive to Q3GA in RASMCs.

The effects of NAC or DPI on PDGF-induced JNK and Akt activations in RASMCs

To confirm that PDGF-induced JNK and Akt activations in RASMCs were mediated by antioxidative effects, RASMCs were pretreated with NAC or DPI for 30 min. As shown in Fig. 5, PDGF-induced JNK and Akt activations were inhibited by NAC (10 mM) or DPI (10 μM) in RASMCs. These results confirmed that JNK and Akt activations by PDGF were mediated by oxidative stress in RASMCs.

Discussion

In the present study, we clarified that Q3GA inhibited PDGF-induced cell migration and proliferation in RASMCs. PDGF-induced JNK and Akt activations were suppressed by Q3GA, but not ERK1/2 and p38 MAP
kinase activations in RASMCs. Furthermore, PDGF-induced cell migration and proliferation were attenuated by a JNK or PI3-kinase inhibitor in RASMCs. From these findings, a circulating form of quercetin in the blood inhibited PDGF-induced RASMC migration and proliferation through the inhibition of JNK and Akt activation. Orally administered quercetin would be converted to its glucuronide form and this form may exert the preventing effects against cardiovascular diseases that are related to VSMC disorders.

Many vascular diseases are characterized by the abnormal accumulation of VSMC in the tunica intima of blood vessels, a process which is thought to occur in part as a result of the migration and proliferation of these cells from the tunica media (15). PDGF is one of the most potent mitogens and chemoattractants for VSMCs and plays the central role in the onset and development of vascular disorders (15, 21). As shown in Figs. 1 and 2, we confirmed that PDGF caused cell migration and proliferation in RASMCs. We also observed that Q3GA inhibited PDGF-induced cell migration and proliferation in RASMCs. A previous study showed that the cholesterol accumulation in the aorta of hypercholesterolemic rabbits was decreased by orally administered quercetin glucoside (30). It was also demonstrated that quercetin metabolites could be detected in the atherosclerotic aorta using high performance liquid chromatography analysis (30). Recently, there was an interesting study reporting that Q3GA specifically accumulates in atherosclerotic lesions in human arteries as determined by using a novel monoclonal antibody 14A2 targeting the Q3GA (31). In addition, we have already observed that Q3GA inhibited Ang II–induced cell hypertrophy in RASMCs (14). Considering the above findings including our present study, it is suggested that VSMC disorders are the potential targets of Q3GA.
in the cardiovascular diseases. It was reported that a
volunteer study clarified that the conjugated metabolites
of quercetin accumulate in human plasma in the
concentration range of $10^{-7} – 10^{-8} \text{M}$ after the periodic
ingestion of onions with meals for 1 week (10). In the
present study, we used Q3GA at 1, 10, and 100 $\mu\text{M}$.
Previous in vitro studies using cultured cells required
relatively higher concentrations ($\geq 10^{5} \text{M}$) as compared with the plasma concentrations (up to $\mu\text{M}$) reported in the human studies (10). Furthermore, in vivo studies to elucidate the precise mechanisms by
which Q3GA exerts potential therapeutic effects on
vascular diseases are needed.

It has been reported that MAP kinases and PI3-kinase are
known to participate in PDGF-induced VSMC
migration and proliferation (23–25). As shown in
Fig. 4, we observed that Q3GA specifically inhibited
PDGF-induced JNK and Akt activation, but not ERK1/2
and p38 MAP kinase activation in RASMCs. These
results were similar to our previous study in which Q3GA inhibited Ang II–induced JNK activation, but not ERK1/2 and p38 MAP kinase activation, in RASMCs (14). Next, we confirmed that 30 $\mu\text{M}$ SP600125, a JNK inhibitor, or 10 $\mu\text{M}$ LY294002, a PI3-kinase inhibitor,
significantly suppressed PDGF-induced RASMC
migration and proliferation (data not shown). These results
confirmed that both migration and proliferation by
PDGF were mediated by JNK and Akt activation in
RASMCs. It was suggested that the inhibitory effects of Q3GA on PDGF-induced JNK and Akt activation led to
the suppression of cell migration and proliferation in
RASMCs. It is well known that PDGF stimulates the
production of ROS in smooth muscle cells (32), which in
turn leads to cell migration (33) and proliferation (34).
We demonstrated that PDGF-induced JNK and Akt activations were inhibited by NAC or DPI in RASMCs.
JNK is well known to be activated by ROS in various
cells (35–37). We have already found that the hydro-
philic vitamin E analog Trolox C and ascorbic acid
inhibited Ang II–induced JNK activation in RASMCs (37).
Additionally, it has been reported that hydrogen
peroxide stimulates Akt phosphorylation, suggesting
that Akt activation is redox-sensitive (38). Our previous
study revealed that Q3GA scavenged DPPH radicals
measured by an EPR method (14). The $\alpha$-dihydroxyl
structure in the B-ring of quercetin, which Q3GA also
possesses, is required to exert maximum free radical
scavenging activity (39, 40). Gathering together these
evidences and our results, it may be reasonable to
speculate that the inhibition of PDGF-induced JNK and
PI3K activation by Q3GA is attributable to their anti-
oxidative effects in RASMCs, although the present work
provided no direct evidence. Further studies are needed
to clarify the entire mechanisms for the antioxidative
effects of Q3GA in VSMCs.

In conclusion, we clarified that Q3GA, an active
metabolite of quercetin, specifically inhibited PDGF-
induced JNK and Akt activations and resultant cell
migration and proliferation in RASMCs. Although most of the in vitro pharmacological studies have been carried out using only the quercetin aglycone form, experiments with the in vivo form of quercetin would be important to elucidate the efficacy of orally administered antioxidants
including quercetin. Q3GA would be an active metabo-
lite of quercetin in plasma and may possess preventing
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