Quercetin Activates an Angiogenic Pathway, Hypoxia Inducible Factor (HIF)-1-Vascular Endothelial Growth Factor, by Inhibiting HIF-Prolyl Hydroxylase: a Structural Analysis of Quercetin for Inhibiting HIF-Prolyl Hydroxylase

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ABSTRACT

We investigated a molecular mechanism underlying quercetin-mediated amelioration of colonic mucosal injury and analyzed chemical structure contributing to the quercetin’s effect. Quercetin up-regulated vascular endothelial growth factor (VEGF), an ulcer healing factor, not only in colon epithelial cell lines but also in the inflamed colonic tissue. VEGF derived from quercetin-treated colon epithelial cells promoted tube formation. The VEGF induction was dependent on quercetin-mediated hypoxia-inducible factor-1 (HIF-1) activation. Quercetin delayed HIF-1α protein disappearance, which occurred by inhibiting HIF-prolyl hydroxylase (HPH), the key enzyme for HIF-1α hydroxylation and subsequent von Hippel Lindau-dependent HIF-1α degradation. HPH inhibition by quercetin was neutralized significantly by an elevated dose of iron. Consistent with this, cellular induction of HIF-1α by quercetin was abolished by pretreatment with iron. Two iron-chelating moieties in quercetin, -OH at position 3 of the C ring and/or -OH at positions 3’ and 4’ of the B ring, enabled the flavonoid to inhibit HPH and subsequently induce HIF-1α. Our data suggest that the clinical effect of quercetin may be partly attributed to the activation of an angiogenic pathway HIF-1-VEGF via inhibiting HPH and the chelating moieties of quercetin were required for inhibiting HPH.

The pathogenesis of both ulcerative colitis and Crohn’s disease is unknown, but these forms of inflammatory bowel disease (IBD) may be associated with an inability of the intestinal mucosa to protect itself from luminal challenges and/or inappropriate repair after intestinal injury (Beck and Podolsky, 1999). In general, the gastrointestinal mucosa has a remarkable ability to repair damage. When the integrity of the superficial mucosa is breached, ulcer healing, a complex and tightly regulated process of filling the mucosal defect, begins (Mammen and Matthews, 2003). Angiogenesis, the formation of new microvessels, is one of the critical components for repair of mucosal defect. This facilitates nutrient and oxygen delivery to the injured area, thus enabling cell proliferation and migration (Tarnawski, 2005). A recent study demonstrates that stimulation of vascular factors with vascular endothelial growth factor (VEGF), a potent angiogenic factor, is sufficient for healing of gastrointestinal ulcers, including IBD (Tarnawski, 2006).

Hypoxia-inducible factor (HIF-1) is a heterodimeric transcription factor composed of HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT, HIF-1β) (Wang et al., 1995). HIF-1α and HIF-1β mRNAs are constantly expressed under normoxic and hypoxic conditions (Wiener et al., 1996). However, HIF-1α protein is significantly increased by hypoxia, whereas the HIF-1β protein remains constant regardless of oxygen tension (Salceda and Caro, 1997). Under normoxia, HIF-1α protein is remarkably unstable, and its degradation by the proteasome is orchestrated by the ubiquitin protein ligase VHL (Salceda and Caro, 1997; Huang et

SUPPLEMENTAL MATERIAL

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ABBREVIATIONS: IBD, inflammatory bowel disease; HIF-1, hypoxia inducible factor-1; NFκB, nuclear factor-κB; HPH, hypoxia inducible factor-prolyl hydroxylase; VEGF, vascular endothelial growth factor; TNBS, 2,4,6-trinitrobenzene-sulfonic acid; VHL, von Hippel Lindau; ARNT, aryl hydrocarbon receptor nuclear translocator; PMSF, phenylmethylsulfonyl fluoride; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; IVT, in vitro-translated; ELISA, enzyme-linked immunosorbent assay; MG-132, N-benzoyloxy carbonyl (Z)-Leu-Leu-leucinal.
al., 1998; Cockman et al., 2000; Tanimoto et al., 2000). Under normoxia, VHL recognizes HIF-1α as a substrate because of the enzymatic modification of HIF-1α by prolyl hydroxylases, whose function is inhibited during hypoxia (Ivan et al., 2001; Jaakkola et al., 2001). Stabilization of HIF-1α by hypoxia or its mimetics is accompanied by its nuclear translocation, heterodimerization with HIF-1β, and transcription of genes encoding proteins functioning to increase angiogenesis and promote cell survival and proliferation that are physiological responses not only for adaptation to hypoxia but also for repair of damaged tissue (Semenza, 1998). In fact, HIF-1 is identified as a critical factor for barrier protection during mucosal insult and, further, plays a key role in healing gastrointestinal ulcer via transactivating VEGF (Baatar et al., 2002; Hashimoto et al., 2004).

Quercetin, the aglycone of rutin, is the most common flavonoid in nature. It has numerous biological activities, including anti-inflammatory effect (Nijveldt et al., 2001). In a previous report, we showed that quercetin is effective in ameliorating the experimental colitis of rats and suggested that a molecular mechanism underlying the clinical effect of quercetin is suppression of a major proinflammatory pathway, tumor necrosis factor dependent NFκB activation (Kim et al., 2005). In this study, we investigated an additional molecular mechanism for the clinical effect of quercetin. Our data demonstrate that quercetin up-regulated an ulcer-healing factor, VEGF, in colon epithelial cells and the inflamed colonic tissue, and quercetin-mediated VEGF induction is dependent on HIF-1 activation, which occurs by quercetin inhibition of HIF-prolyl hydroxylase. Furthermore, it was revealed that two chelating moieties in quercetin were required for inhibiting the enzyme and consequently activating HIF-1. These results imply that, in addition to suppressing an inflammation-provoking factor, NFκB pathway, quercetin activation of an angiogenic pathway, HIF-1-VEGF, contributes to the clinical effect of quercetin on experimental colitis.

Materials and Methods

Chemicals and Animals. 3,5,7,3′,4′-Pentahydroxyflavone (quercetin), 3-hydroxyflavone, and 2,4,6-trinitrobenzene-sulfonic acid (TNBS) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 5-Hydroxyflavone and 3′,4′-dihydroxyflavone, methylated quercetin derivatives, were purchased from Indofine Chemical Co. Inc. (Hillsborough, NJ). 2-Ketoglutatarate, ascorbate, (+/-)-6-O-isopropylidene-1-ascorbic acid, and ferrous chloride were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent-grade, commercially available products. Male Sprague-Dawley rats (240–260 g, 8 weeks old) were purchased from Daehan Biotec Co. Ltd. (Daeju, Korea) and housed in the animal care facility at Pusan National University (Busan, Korea). The animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Cell Culture and Transient Transfection. Human colon epithelial cell lines HCT116 and SW620 (American Type Culture Collection, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin/streptomycin (Biofluids). For transient transfection of plasmids, 293 cells were plated in 6-cm dishes to be 50 to 60% confluent on the day of cotransfection with Flag-VHL (5 μg; a gift from Dr. J. Issacs, Medical University of South Carolina, Charleston, SC), HA-HIF-1α plasmid (5 μg; a gift from Dr. L. Neckers, National Cancer Institute, Bethesda, MD), and CMV Renilla reniformis luciferase plasmid (4 ng; Promega, Madison, WI). Fugene (Roche, South San Francisco, CA) was used as a transfection reagent. One day after transfection, cells were treated with each reagent as indicated in the figure legends.

Immunoblot Analysis and Immunoprecipitation. Cells were lysed, and nuclear extracts were prepared as described previously (Andrews and Fuller, 1991). To prepare tissue nuclear extracts, the inflamed distal colon was removed and mixed with 5-fold amount of buffer C (10 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 0.3 μM aprotinin, 1 μM pepstatin, and 1 mM PMSF) followed by homogenation. Nonidet P-40 (10%) was added to the homogenates at the ratio of 50 μM/ml after 20-min incubation in ice, and the mixture was vortexed vigorously for 15 s and centrifuged at 14,000 rpm and 4°C for 3 min to afford the nuclear pellets. After removing the supernatants, an appropriate volume of buffer containing 20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 0.3 μM aprotinin, 1 μM pepstatin, and 1 mM PMSF was added to the nuclear pellets, and the tubes were rotated on a small rotatory shaker at 4°C for 20 min followed by centrifugation at 14,000 rpm and 4°C for 10 min. Protein concentration in the supernatants was determined by the BCA method. The nuclear extracts were transferred to a fresh tube and stored at −70°C until used. Cell or tissue nuclear extracts were electrophoretically separated using 7.5 or 10%. Proteins were transferred to nitrocellulose membranes (Protran; Whatman Schleicher and Schuell, Keene, NH), and HIF-1α protein was detected in nuclear extracts using a monoclonal anti-HIF-1α antibody (Transduction for human HIF-1α or Novus for murine HIF-1α). Peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was used at a dilution of 1:1000. Signals were visualized using the SuperSignal chemiluminescence substrate (Pierce, Rockford, IL). Experiments were performed in duplicate and normalized with antibodies to topoisomerase II (Santa Cruz Biotechnology, Santa Cruz, CA). For immunoprecipitation, 293 cell lysates (0.7 mg of protein), prepared as described in the previous section, were incubated with 20 μl of anti-HA antibody bound beads (Covance Research Products, Berkeley, CA). The beads were washed five times with lysis buffer, resuspended in 1× SDS sample buffer, and boiled for 5 min. Immunoprecipitated proteins were separated by 10% SDS-PAGE. Immunoblot analysis was done as aforementioned.

In Vitro VHL Capture Assay. Biotinylated wild-type or proline-hydroxylated peptides (corresponding to HIF residues 556–574) were synthesized (American Peptide Company, Sunnyvale, CA), dissolved in sterile water (500 μg/ml), and incubated with streptavidin beads (Pierce ImmunoPure) at 4°C for 2 h. The beads were washed twice with VHL binding buffer (20 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) and three times with reaction buffer (20 mM Tris, pH 7.5, 5 mM KCl, 1.5 mM MgCl2, and 100 μM dithiothreitol). For each condition, 2 μg of peptide/20 μl of beads was aliquoted into separate tubes, and reaction buffer was added, along with cofactors (100 μM 2-ketogluartic acid, 100 μM L-ascorbic acid, and 50 μM ferrous chloride). The beads and HPH cofactors were mixed at room temperature for 15 min in reaction buffer. Before this incubation, any inhibitors or competing factors were added to the appropriate tubes. Separate in vitro-translated (IVT) reactions (Promega) were the source for the HIF prolyl hydroxylase protein (HPH-2 plasmid) kindly provided by S. McKnight, University of Texas Medical Center, Dallas, TX) and Flag-VHL (Issacs et al., 2002). A 5-μl aliquot of IVT HPH-2 was added to the bead-peptide mixture for 1 h at 30°C. Afterward, the beads were washed with VHL binding buffer and 10 μl of Flag-VHL IVT was added to the beads overnight at 4°C. The beads were washed, SDS Laemmli buffer was added, the samples were boiled, subjected to SDS-PAGE, and resultant blots were probed for Flag.

VEGF Analysis. Cells were treated as indicated in the figure legends. Medium was collected after 10-h treatment. A VEGF ELISA kit (R&D Systems, Minneapolis, MN) was used to assess secreted VEGF levels from a 200-μl aliquot of medium. Each sample was
harvested for protein, which was used to normalize VEGF levels. An experiment for each condition was carried out in triplicate. To measure VEGF level in the tissue, the inflamed distal colon was removed and mixed with 5-fold amount of buffer C (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.3 μM aprotinin, 1 μM peptatin, and 1 mM PMSF) followed by homogenization. The homogenates were centrifuged at 2000 rpm and 4°C for 2 min. The supernatant (100 μl) was transferred to a fresh microtube and then was centrifuged again at 14,000 rpm and 4°C for 10 min. An appropriate volume of the supernatant was subjected to VEGF ELISA.

Tube Formation Assay. HUVEC cells (5 x 10⁵ cells) were seeded on a layer of previously polymerized Matrigel. The culture supernatants (1/10 volume of HUVEC media) obtained from HCT cells, left untreated or treated with quercetin (25 μM), were added to each well and incubated at 37°C. VEGF was used as a positive control. After 24 h, changes of cell morphology were captured through a phase-contrast microscope and photographed. The same experiment was done in the presence of a VEGF-neutralizing antibody (1 μg/ml, R&D Systems).

TNBS-Induced Inflammation. Inflammation was induced by the method of Morris et al. (1989) and Yano et al. (2002). In brief, before induction of colitis, rats were starved for 24 h but had free access to water. The rats were lightly anesthetized with ether. A rubber cannula (outer diameter, 2 mm) was inserted rectally into the colon such that the tip was 8 cm proximal to the anus, approximately at the splenic flexure. TNBS dissolved in 50% (v/v) aqueous ethanol was instilled into the colon via the rubber cannula (15 mg/0.3 ml/rat).

Data Analysis. Results were expressed as means ± S.E. The statistical differences among the results of the various groups were compared by the Student's t test. A value of p < 0.05 was considered significant.

Results

Quercetin Up-Regulates VEGF in Human Colon Epithelial Cells and the Inflamed Colonic Tissue of Rats. We reported previously that quercetin is effective in ameliorating TNBS-induced rat colitis, and the clinical effect is elicited by at least partly inhibiting a major pro-inflammatory NFκB pathway (Kim et al., 2005). To explore an additional pharmacological mechanism of the quercetin effect on experimental colitis, we investigated whether quercetin, in addition to suppressing an inflammatory signal, could stimulate a tissue repair signal, which accelerates healing of inflammatory injury. Because quercetin is able to induce VEGF in endothelial cells (Wilson and Poellinger, 2002), and
VEGF promotes angiogenesis, a critical step for tissue repair, we examined whether quercetin increased secretion of VEGF in human colon epithelial cells. Cells were treated with quercetin for 10 h. The cell culture supernatants were subjected to VEGF ELISA. As shown in Fig. 1A, quercetin induced VEGF in the cells in a dose-dependent manner. To see whether quercetin is also able to induce VEGF in the inflamed colonic tissue, a 300-μl aliquot of quercetin (50 and 100 μM) in pH 6.8 phosphate-buffered saline buffer was administered to the inflamed site through rectal route once a day starting from 1 day after induction of inflammation by TNBS. The inflamed colonic tissues were removed and homogenized 4 h after the last (sixth) treatment with quercetin followed by centrifugation. VEGF levels in the supernatants were measured using a VEGF ELISA kit. As shown in Fig. 1B, quercetin increased VEGF levels in the inflamed colonic tissue up to approximately 2-fold.

**VEGF Induction by Quercetin Is Dependent on HIF-1.** Because VEGF is a target gene of HIF-1 (Semenza, 1998), we examined whether VEGF induction by quercetin was mediated by HIF-1. First, we tested whether quercetin was able to up-regulate HIF-1α protein in cells and the inflamed tissue. Colon epithelial cells were treated with quercetin and were lysed to obtain nuclear extracts. Colon epithelial cells were treated with quercetin and were lysed to obtain nuclear extracts. HIF-1α levels were examined by Western blot. As shown in Fig. 2A, quercetin up-regulated HIF-1α in the colon epithelial cells in a dose-dependent manner. To examine HIF-1α induction by quercetin in the inflamed colonic tissue, quercetin was administered rectally after induction of inflammation as aforementioned. The inflamed distal colon was subjected to nuclear extraction, and HIF-1α levels in the nuclear extracts were monitored by Western blot. As shown in Fig. 2B, HIF-1α protein was up-regulated in the inflamed colonic tissue. These results suggest that HIF-1α up-regulation is correlated with VEGF induction upon quercetin treatment. To further clarify that quercetin induction of VEGF is dependent on HIF-1, quercetin was treated in hepa1c1c7 cells that contain wild-type ARNT and in matched hepa1c4 cells that

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**Fig. 2.** Quercetin induction of VEGF is dependent on HIF-1. A, colon epithelial cells (HCT116 and SW620) were treated with various concentrations of quercetin for 4 h, and HIF-1α levels were monitored in the nuclear extracts as described under Materials and Methods. B, the tissue pellets that were obtained from Fig. 1B were subjected to nuclear extraction. HIF-1α levels were monitored in the nuclear extracts as described under Materials and Methods. C, hepa1c1c7 cells (H1C1C7) were treated with quercetin (50 μM) or hypoxia for 10 h, and VEGF in the cell culture supernatants and HIF-1α in the nuclear extracts were analyzed as described under Materials and Methods. The same experiment was done using hepa1c4 cells (H1C4) with genetically defective ARNT (except for immunodetecting HIF-1α). The data are means ± S.E. (n = 3). *, P < 0.01 versus control. D, HUVEC cells (5 × 10^5 cells) were seeded on a layer of previously polymerized Matrigel. The cell culture supernatants (1/10 volume of HUVEC media) obtained from HCT116 cells, left untreated or treated with quercetin (25 μM), were added to each well and incubated at 37°C. VEGF was used as a positive control. After 24 h, changes of cell morphology were captured through a phase-contrast microscope and photographed. The same experiment was done in the presence of a VEGF-neutralizing antibody (VEGF-Ab).
are unable to transactivate HIF-1-dependent genes because of a genetic defect in ARNT (Li et al., 1996), and ELISA and Western blotting were performed to detect VEGF in the cell culture supernatants and HIF-1α protein in the nuclear extracts. Hypoxia (approximately 1% O₂) was used as a positive control for VEGF induction. As shown in Fig. 2C, hypoxia and quercetin induced VEGF only in hepa1c1c7 cells. Furthermore, this increase in VEGF secretion was correlated with an increase in HIF-1α level. However, in ARNT-deficient hepa1c1c4 cells, quercetin failed to increase VEGF secretion, thereby demonstrating that quercetin-mediated VEGF induction is dependent on transcriptionally active HIF-1. Because quercetin activated HIF-1-VEGF, an angiogenic pathway, we wondered whether quercetin could promote angiogenesis via the pathway. To examine this, HCT116 cells were treated with quercetin for 10 h, and the cell culture supernatants were added to the wells in which endothelial cells were seeded, and the tube formation was measured. VEGF was used as a positive control. To see whether quercetin-induced VEGF is the main factor for the in vitro tube formation, the same experiment was carried out in the presence of a VEGF-neutralizing antibody. As shown in Fig. 2D, quercetin-induced VEGF was significantly reduced in the presence of the antibody. The cell culture supernatant obtained from HCT 116 cells left untreated with quercetin showed tube formation similar to that of a control (no addition of the cell culture supernatant), and the VEGF antibody did not significantly affect tube formation in this condition. On the contrary, the cell culture supernatant obtained from quercetin-treated HCT116 cells enhanced tube formation, which was inhibited significantly by pretreatment with the VEGF antibody. Quantitative analysis of tube formation is shown in Supplemental Data 1A. In vitro cell proliferation assay was also done with the cell culture supernatants. As shown in Supplemental Data 1B, proliferation of HUVEC cells was promoted by the supernatant obtained from quercetin-treated cells, which was comparable with that by VEGF.

**Quercetin Up-Regulates HIF-1α by Inhibiting HIF Prolyl Hydroxylase.** Because the α-subunit of HIF is tightly regulated at the post-translational level by protein degradation (Huang et al., 1998), we considered whether quercetin modulated HIF-1α stability. HCT116 cells were either left untreated or were pretreated with quercetin for 4 h followed by the addition of the protein synthesis inhibitor cycloheximide, for the indicated times and disappearance rate of HIF-1α protein was compared. As shown in Fig. 3A, HIF-1α protein was extremely labile, disappearing in 5 min in cells left untreated with quercetin. In marked contrast, substantial amount of HIF-1α protein still remained in quercetin-pretreated cells 40 min after the addition of cycloheximide, suggesting that quercetin stabilized HIF-1α protein. The central molecular mechanism for regulating HIF-1α protein stability is VHL-dependent proteasomal HIF degrada-

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**Fig. 3.** Quercetin up-regulates HIF-1α by inhibiting HIF prolyl hydroxylase. A, HCT116 cells were either left untreated or were pretreated with quercetin (50 μM) for 4 h, followed by the addition of cycloheximide for the indicated times. Levels of HIF-1α were visualized from nuclear extracts. Blots were reprobed for Topo II expression as a control for equivalent loading within each group. B, renal carcinoma cells that are deficient for VHL function (UMRC2) or a clonally selected line with VHL stably expressed (UMRC2/VHL) was treated with quercetin (50 μM) for 4 h, and HIF-1α protein was detected in nuclear extracts. C, a VHL capture assay using biotinylated HIF peptide was performed as described under Materials and Methods. Left, the assay was performed in the presence of cofactors and the indicated concentrations of quercetin, and resultant blots were probed for Flag (VHL). The control lane (con) represents the assay in the absence of added cofactors, whereas the untreated (UT) lane contains all required cofactors. Right, the same assay was repeated using a chemically hydroxylated peptide and increasing concentrations of quercetin. D, 293 cells, cotransfected with HA-HIF-1α and Flag-VHL, were treated with quercetin in the presence of MG-132 and were lysed 4 h later. HA-HIF-1α protein was immunoprecipitated by the addition of anti-HA antibody-bound beads. Immunoprecipitated proteins were solubilized in SDS sample buffer and separated by SDS-PAGE. Blots were probed with an anti-Flag antibody or an anti-HA antibody.
tion after hydroxylation of proline residues in HIF-1α by HPH (Ivan et al., 2001; Jaakkola et al., 2001). We considered whether quercetin might affect the HIF-regulating pathway. To test this, we first examined the effects of quercetin upon HIF-1α expression in either the parental VHL-deficient renal carcinoma cell line UMRC2, or UMRC2/VHL, which expresses a stably integrated construct encoding Flag-VHL (Isaacs et al., 2002). As shown in Fig. 3B, quercetin increased HIF-1α expression in UMRC2/VHL; however, when this experiment was repeated in the VHL-deficient parental line, quercetin was unable to induce HIF-1α expression. Although these data support an involvement of VHL in quercetin-mediated HIF induction, it remained unclear how quercetin intervenes in VHL-dependent HIF-1α regulation. Because HIF-prolyl hydroxylase is the key enzyme for VHL-dependent HIF degradation, we examined whether quercetin affected HIF-prolyl hydroxylase activity. To do this, we used an in vitro VHL capture assay (Bruick and McKnight, 2001; Isaacs et al., 2005) with a biotinylated HIF peptide that contains a conserved proline residue subject to HPH-dependent hydroxylation. As shown in Fig. 3C (left), the association of VHL with the HIF peptide in the absence of exogenously added cofactors (control lane) is undetectable. When the required cofactors for HPH are added (UT lane), the association between the HIF peptide and VHL is markedly enhanced. It is striking that a 25 μM concentration of quercetin significantly reduced the association between HIF and VHL, and a 100 μM concentration completely abrogated the interaction between these proteins. Finally, we used a chemically synthesized hydroxylated peptide to verify that quercetin directly affects HPH activity and does not impair VHL protein. As shown in Fig. 3C (right), quercetin does not impair the ability of VHL to associate with hydroxylated HIF peptide up to 200 μM. In contrast, an HIF peptide in which the two proline residues were mutated to alanine failed to bind VHL under any circumstances (data not shown). Our data strongly support the premise that quercetin is a potent inhibitor of HPH. To test this notion in cells, we transfected an HA-HIF-1α and a Flag-VHL plasmid in 293 cells followed by 4-h treatment with a proteasome inhibitor MG-132 in the presence or absence of quercetin. After immunoprecipitation with an anti-HA antibody, VHL levels in the immunocomplexes were monitored using an anti-Flag antibody. As shown in Fig. 3D, MG-132 increased the VHL level in the immunocomplex, and, consistent with the result of VHL capture assay, quercetin effectively prevented the increase of the VHL level.

Two Chelating Moieties of Quercetin Are Involved in Inhibiting HPH. Our data demonstrate that quercetin inhibited HPH. We wished to explore how quercetin inhibited the enzyme. Because the enzyme requires cofactors to catalyze hydroxylation of HIF-1α, we examined whether quercetin impaired the activity of the enzyme by affecting availability of the required factors. In vitro VHL capture assay was performed in the presence of various concentrations of the factors. As shown in Fig. 4A (top), whereas 10-fold increase of either 2-ketoglutarate or ascorbate did not at all affect the inhibitory effect of quercetin on the enzyme (data not shown), an escalating dose of iron attenuated the quercetin effect, as represented by restored VHL association. To further test this, cells were treated with quercetin in the presence of 200 μM iron, and HIF-1α level was monitored. Consistent with the in vitro result, HIF-1α induction was abolished in the iron-enriched condition (Fig. 4A, bottom). This result suggests that quercetin inhibits HPH activity by reducing the availability of iron. Quercetin reportedly is able to form metal complexes through three chelating moieties (Morel et al., 1994). To examine which chelating moiety of quercetin is required for inhibiting HPH, we treated cells with quercetin derivatives, namely 3′,4′-hydroxyflavone, 3-hydroxyflavone, and 5-hydroxyflavone. As shown in Fig. 4B (top), 3′,4′-hydroxyflavone and 3-hydroxyflavone but not 5-hydroxyflavone elevated the HIF-1α level. In addition, cells were treated with either 3′,4′-hydroxyflavone or 3-hydroxyflavone in the iron-enriched condition. HIF-1α induction was abolished completely (Fig. 4B, middle), indicating that iron chelation via the moieties resulted in HIF-1α induction. To further test the requirement of the chelating ability for HIF-1α induction, cells were treated with the methylated forms of the quercetin derivatives with no chelating activity, and HIF-1α level was monitored. As shown in Fig. 4B (lower), the methylated derivatives failed to induce HIF-1α. To ensure that the quercetin derivatives up-regulated HIF-1α via inhibiting HPH, VHL capture assay was carried out with the quercetin derivatives. As shown in Fig. 4C, as expected, 3′,4′-hydroxyflavone decreased VHL association in a dose-dependent manner. It is interesting that although 3-hydroxyflavone increased the HIF-1α level in cells, it did not show an ability to attenuate VHL association, which represents no inhibition of HPH. We speculated that 3-hydroxyflavone might inhibit the enzyme by antagonizing interaction of the catalytic iron with ascorbate or 2-ketoglutarate in cells in which concentrations of them may be lower than those for the in vitro assay. In the process of HIF-1α hydroxylation by HPH, the transient association of the catalytic iron with ascorbate or 2-ketoglutarate is required (Kivirikko et al., 1989; Hewiston and Schofield, 2004). To test this possibility, we carried out the in vitro assay without exogenous addition of either ascorbate or 2-ketoglutarate. As reported previously (Ivan et al., 2002), HPH was still active and induced VHL association in the condition (data not shown). As shown in Fig. 4D, consistent with our hypothesis, 3-hydroxyflavone effectively reduced VHL association in the absence of exogenous ascorbate, and furthermore, the addition of ascorbate recovered VHL association. This phenomenon was not observed in the case of 2-ketoglutarate (data not shown). To further test the ascorbate effect, cells were treated with 3-hydroxyflavone in the presence of ascorbate or a cell-permeable ascorbate, and HIF-1α was monitored. As shown in Fig. 4E, pretreatment with ascorbate nullified the effect of 3-hydroxyflavone on HIF-1α induction.

**Discussion**

In this study, we demonstrate that quercetin, the aglycone of rutin, induced an ulcer-healing factor, VEGF, via activating the HIF-1 pathway most likely by inhibiting HIF-prolyl hydroxylase. Furthermore, we provide information on the structural requirement of quercetin for inhibiting the enzyme. Quercetin was reported to stabilize HIF-1α protein and induce VEGF in endothelial cells (Wilson and Poellinger, 2002). In line with the report, we found that quercetin showed the same effect in colon epithelial cells and the inflamed colonic tissue. Moreover, we clarified HIF-1 depen-
idence in quercetin-mediated VEGF induction by showing that quercetin was able to induce VEGF in normal mouse hepatoma cells but not in the matched cells with genetically defective HIF-1α and consequent inactivation of HIF-1 pathway. Furthermore, we elucidated the molecular mechanism underlying quercetin-mediated HIF-1α up-regulation and subsequent activation of HIF-1 pathway. Our data showing that quercetin delayed the degradation of HIF-1α protein and quercetin-mediated HIF-1α induction occurred only in cells with functional VHL suggest that the flavonoid stabilizes HIF-1α protein by preventing VHL-dependent HIF-1α degradation. This hypothesis is validated by providing compelling evidence that quercetin inhibited HPH, thus interfering with the hydroxylation of HIF-1α, a critical post-translational modification for VHL-dependent degradation of HIF-1α. In the in vitro VHL capture assay, in which in vitro-translated HPH hydroxylates its substrate, HIF-peptide, and subsequently the hydroxylated peptide associates with in vitro-translated VHL, we observed that the addition of quercetin before reaction of HPH with HIF peptide attenuated VHL association with HIF peptide, indicating reduced hydroxylation of HIF peptide by quercetin-mediated HPH inhibition. This in vitro result was confirmed by demonstrating that quercetin decreased the level of VHL precipitated together with HIF-1α in cells. We suggest that HPH inhibition by quercetin occurred via reducing the availability of iron, an essential cofactor of the enzyme. This argument was supported by the data showing that 1) escalation of iron dose attenuated the quercetin effect on VHL association; 2) pretreatment with iron prevented HIF-1α protein induction by quercetin; and 3) dose changes of the other factors, ascorbate and 2-ketoglutarate, did not influence the quercetin effects on VHL association. In line with this finding, it was revealed that chelating moieties of quercetin were involved in HPH inhibition. Although quercetin (3,5,7,3′,4′-pentahydroxyflavone) has three chelating moieties in it, two of them seem to be used to reduce the availability of iron, as demonstrated in the data showing that 3′,4′-dihydroxyflavone and 3-hydroxyflavone but not 5-hydroxyflavone decreased VHL association with HIF-1 peptide and elevated HIF-1α level. Furthermore, the methylated forms of 3′,4′-dihydroxyflavone and 3-hydroxyflavone, which are not able to form an iron complex, lost the ability to decrease the VHL association (Supplemental Data 1A) and elevate HIF-1α level; furthermore, pretreatment with iron neutralized the effects of the two flavones, 3′,4′-dihydroxyflavone and 3-hydroxyflavone (Supplemental data 1B). Because, for hydroxylation of the substrate HIF-1α by HIF prolyl hydroxylase, iron in the catalytic site needs to be associated transiently with the required factors, 2-ketoglutarate and ascorbate (Kivirikko et al., 1989; Hewitson and

![Fig. 4](https://molpharm.aspetjournals.org/)

Two chelating moieties of quercetin enable the flavonoid to inhibit HPH and subsequently induce HIF-1α. A, top, VHL capture assay was performed in the presence of escalating dose of iron and quercetin (50 μM), and resultant blots were probed for Flag (VHL). Bottom, HCT cells were treated with quercetin (50 μM) in the presence or absence of various ferrous chloride, and HIF-1α protein levels were monitored in the nuclear extracts. B, top, HCT116 cells were treated with quercetin derivatives (50 μM), 3-hydroxyflavone (3-HF), 5-hydroxyflavone (5-HF), or 3′,4′-dihydroxyflavone (DHF), and HIF-1α protein levels were monitored in the nuclear extracts. Middle, HCT116 cells were treated with 3-hydroxyflavone (50 μM) or 3′,4′-dihydroxyflavone (50 μM) in the presence or absence of ferrous chloride (200 μM), and HIF-1α protein levels were monitored in the nuclear extracts. Bottom, HCT116 cells were treated with 3-hydroxyflavone (50 μM), 3′,4′-dihydroxyflavone (50 μM), or their methylated forms, 3-methoxyflavone (100 μM, 3-MHF) or 3′,4′-dimethoxylavone (100 μM, DMF) and HIF-1α protein levels were monitored in the nuclear extracts. C, VHL capture assay was performed in the presence of 3-hydroxyflavone (50 μM), 3′,4′-dihydroxyflavone (50 μM), and resultant blots were probed for Flag (VHL). D, VHL capture assay was performed with 3-hydroxyflavone (50 μM) in the presence or absence of 3-hydroxyflavone (100 μM), and resultant blots were probed for Flag (VHL). E, VHL capture assay was performed with 3-hydroxyflavone (50 μM) in the presence or absence of either ascorbate (V.C, 5 mM) or a cell-permeable ascorbate, (+)-5,6-O-Isopropylidene-L-ascorbic acid (Iso V.C, 1 mM), and HIF-1α protein levels were monitored in the nuclear extracts.
Schofield, 2004), it is likely that the iron chelation with the flavones prevents the association, thus resulting in impairing the catalytic activity of HPH. This hypothesis is supported by our observation that 3-hydroxyflavone inhibition of HPH and 3-hydroxyflavone induction of HIF-1α were abolished completely by the addition of ascorbate. In the case of 3',4'-dihydroxyflavone, the HPH inhibition was not recovered in the presence of excess amount of 2-ketoglutarate or ascorbate (Supplemental Data 1C), suggesting that the affinity between the flavone and iron is too strong to be replaced with the required factors, or the HPH inhibition occurs by a way regardless of antagonizing the required factors. Although it is not clear which of the two chelating moieties of quercetin associates preferentially with the iron in the enzyme catalytic site, 3',4'-dihydroxy group should be the preferential partner for the iron chelation in the condition in which ascorbate exists over the concentration for the antagonism. In fact, the observation that quercetin still inhibits HPH in the presence of 1 mM ascorbate manifests the situation.

In our previous report, we suggested that quercetin-mediated amelioration of rat colitis was elicited by inhibiting NFκB activity (Kim et al., 2005). Our data demonstrating quercetin activation of HIF-1-VEGF pathway in cells and inflamed colonic tissue suggest that quercetin exerts its clinical effect by not only inhibiting the inflammatory signal but also activating the tissue repair signal. Although the activation of HIF-1-VEGF pathway promotes angiogenesis that could enhance repair of injured mucosa, quercetin per se was reported to inhibit growth of endothelial cells (Tan et al., 2003), thus possessing antiangiogenic potential at the concentration range used in this experiment. However, it is more likely that quercetin exerts a positive effect on angiogenesis in the colitis rat model. This is based on the following reasons: 1) unlike the previous report in which endothelial cells were exposed continually to quercetin for 1 to 3 days, quercetin in the large intestine remained at 200 to 30 μM for approximately 6 h after oral administration of a glycoside of quercetin (Kim et al., 2005), which may be enough for quercetin to activate HIF-1-VHL pathway but not to inhibit growth of endothelial cells; 2) because concentration of quercetin diffused to the colonic endothelial cells should be lower than that of quercetin in the gut lumen; and 3) considering the second reason stated above, the tube formation assay, in which the diluted supernatant of quercetin-treated cells was used, may reflect the action of quercetin in the inflamed large intestine and, indeed, VEGF in the diluted supernatant promoted tube formation of HUVEC cells.

Although the exact molecular mechanism by which quercetin inhibits growth of endothelial cells is not known yet, a number of reports suggest that quercetin elicits the cytotoxic effect by blocking cell proliferation signals such as phosphatidylinositol-3 kinase (Agullo et al., 1997; Williams et al., 2004). According to recent reports on the relationship between flavonoid structure and phosphatidylinositol-3 kinase or NFκB (Agullo et al., 1997; Chen et al., 2004), whereas the double bond (C2–C3) of the C ring is important for both biological activities, the effect of hydroxyl moiety (–OH) of flavonoids on the protein targets varies depending on position and number of the moiety on the flavonoid skeleton. In addition to this structural information, our data on the structural requirement of quercetin for HPH inhibition may provide a possibility to separate antiangiogenic activity from biological activities of quercetin (for amelioration of IBD) via structural modification. Moreover, our data add one to the structural criteria that could be used to predict biological activities of flavonoid.

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