

## The Dietary Flavones Apigenin and Luteolin Impair Smooth Muscle Cell Migration and VEGF Expression through Inhibition of PDGFR- $\beta$ Phosphorylation

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**Abstract** Platelet-derived growth factor (PDGF)-dependent recruitment of mural cells such as pericytes and smooth muscle cells plays a central role in the maturation and stabilization of newly formed vasculature during angiogenesis. In this work, we show that the dietary flavones apigenin and luteolin may interfere with this event through their inhibitory effect on PDGF-dependent phosphorylation of PDGF receptor  $\beta$  (PDGFR- $\beta$ ) in smooth muscle cells. Inhibition of PDGFR- $\beta$  activity by apigenin and luteolin occurred at low concentrations of the molecules and resulted in the inhibition of extracellular signal-regulated kinase and Akt phosphorylation triggered by PDGF, as well as in a marked reduction of the migratory and invasive properties of these cells. Apigenin and luteolin also strongly inhibit the PDGF-dependent increase in vascular endothelial growth factor (VEGF) mRNA levels and the secretion of VEGF by smooth muscle cells as well as vessel formation in the mouse Matrigel plug assay, suggesting that the inhibitory effects of both molecules on smooth muscle cell function result in impaired angiogenesis. Overall, these results identify apigenin and luteolin as dietary-derived inhibitors of PDGFR- $\beta$  activity and suggest that this inhibitory effect may contribute to the chemopreventive properties of these molecules.

Most solid tumors induce a neovascular response to acquire the nutrients and oxygen essential for the formation, growth, and dissemination of cancer (1). During this process, known as tumor angiogenesis, tumor cells secrete angiogenic cytokines such as vascular endothelial growth factor (VEGF) that induce proliferation and migration of endothelial cells, leading to the formation of a new vascular network (2). As a result, inhibition of VEGF-mediated signaling has become a primary target for antiangiogenic strategies, and inhibitors directed against either VEGF or its receptor, VEGF receptor 2, prevent vascularization and the growth of a large number of experimental tumor types (3).

In addition to the central role of VEGF in angiogenesis, the formation of mature vascular networks requires the recruitment of mural cells (pericytes and smooth muscle cells) that

stabilize the newly formed vasculature (4). There is increasing evidence that secretion of platelet-derived growth factor (PDGF-B) by endothelial cells and tumor cells and stimulation of its cognate receptor PDGFR- $\beta$  associated with vascular smooth muscle cells/pericytes are crucial events for the stabilization of the capillary wall (5, 6). The importance of this process was exemplified by studies showing that pericyte deficiency in mice lacking PDGF-B or PDGFR- $\beta$  promotes several microvascular defects such as capillary dilation (microaneurysm), leakage, and rupture, leading to lethal hemorrhage and edema (7, 8). In addition, smooth muscle cells that surround tumor vessels produce VEGF, thereby promoting endothelial cell survival (9), and this paracrine system seems to be important for the maintenance of tumor vessels because combined inhibition of VEGF and PDGF receptors showed enhanced antiangiogenic activity and caused regression of established tumors (10, 11). The identification of molecules that interfere with PDGF signaling at the smooth muscle cell levels may thus be of considerable importance for the development of novel chemopreventive and chemotherapeutic approaches.

Numerous studies have linked abundant consumption of foods of plant origin to a substantial reduction in risk of developing various cancers (12), a chemopreventive effect that is related to the high content of these foods in several phytochemicals with potent anticancer properties (13). Among these, polyphenols such as flavonoids are found at high levels in several fruits and vegetables as well as in

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beverages such as tea, cocoa, and wine (14). Flavonoids are commonly divided into six subclasses, based on the connection position of the B and C rings and the degree of saturation and hydroxylation of the C ring, as flavonols, flavones, flavanones, flavan-3-ols, isoflavones, and anthocyanidins (15). The mean dietary intake of flavonoids is in the order of a few hundreds of milligrams per day (15), and epidemiologic studies have suggested that high intake of these molecules may be correlated with a decreased risk of several types of cancers (16–20).

Apigenin (4',5,7-trihydroxyflavone) and luteolin (3',4,5,7-tetrahydroxyflavone; Fig. 1) are naturally occurring plant flavones abundantly present in common vegetables and herbs such as parsley, celery, and sweet peppers (21). Although these molecules have been shown to possess numerous anti-inflammatory, antiangiogenic, and anticarcinogenic effects in cell culture and in various animal models (22–25), the mechanisms underlying their chemopreventive effects still remain incompletely understood. In this study, we present evidence that apigenin and luteolin act as potent inhibitors of PDGFR- $\beta$  activities, leading to an inhibition of PDGF-BB-induced smooth muscle cell migration and invasion and to a reduction of VEGF expression by these cells, and that these effects may contribute to the antiangiogenic properties of these molecules.

## Materials and Methods

### Materials

Cell culture media were obtained from Life Technologies and serum was purchased from HyClone Laboratories. Flavonoids (apigenin, luteolin, kaempferol, and myricetin) were obtained from Extra-

synthese S.A. Co. Electrophoresis reagents were purchased from Bio-Rad. Protein A-Sepharose and Protein G-Sepharose were obtained from Amersham Pharmacia Biotech. The anti-PDGFR- $\beta$  (958), anti-phosphospecific(Y857) PDGFR- $\beta$ , and anti-extracellular signal-regulated kinase (ERK)-1/2 (K-23) polyclonal antibodies and the anti-phosphotyrosine (PY99) monoclonal antibody were from Santa Cruz Biotechnologies. Anti-phospho-Akt (Ser<sup>473</sup>), anti-Akt (pan), and anti-phospho-p44/42<sup>MAPK</sup> (Thr<sup>202</sup>/Tyr<sup>204</sup>) polyclonal antibodies were from Cell Signaling Technology. Antimouse and antirabbit horseradish peroxidase-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories and enhanced chemiluminescence reagents were from Perkin-Elmer Life Sciences. Human recombinant fibroblast growth factor (FGF)-2 was obtained from Upstate. Human recombinant PDGF-BB and VEGF were obtained from R&D Systems. Micro bicinchoninic acid protein assay reagents were from Pierce. All other reagents were from Sigma-Aldrich Canada.

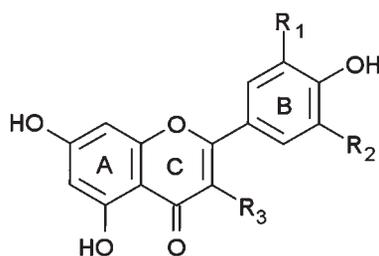
### Inhibition of PDGFR- $\beta$ phosphorylation and downstream signaling pathways by flavonoids

Pulmonary aortic smooth muscle cells were purchased from Clonetics and cultured in smooth muscle growth medium-2 (Clonetics) at 37°C under a humidified 95%-5% (v/v) mixture of air and CO<sub>2</sub>. Confluent pulmonary aortic smooth muscle cells were incubated for 18 h in 0.5% fetal bovine serum containing either vehicle (DMSO) or flavonoids and stimulated with 50 ng/mL PDGF-BB for 5 min. Cells were washed and solubilized on ice in lysis buffer [150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 0.5% (v/v) NP40, and 1% (v/v) Triton X-100], and the resulting lysates were processed for immunoprecipitation studies as previously described (26). Briefly, lysates (200  $\mu$ g protein) from each sample were incubated in lysis buffer overnight at 4°C in the presence of 1  $\mu$ g/mL of anti-PDGFR- $\beta$  antibodies, and immune complexes were collected by incubating the mixture with 25  $\mu$ L (50% suspension) of Protein A-Sepharose beads for 2 h. Bound proteins were solubilized in 2-fold concentrated Laemmli sample buffer [125 mmol/L Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10%  $\beta$ -mercaptoethanol, and 0.00125% bromophenol blue], boiled for 4 min, and resolved by SDS-PAGE (7.5% gel). For analysis of ERK and Akt phosphorylation, lysates from control and treated cells were solubilized in Laemmli sample buffer, boiled for 4 min, and resolved on 10% SDS-PAGE.

Following electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes, blocked overnight at 4°C with TBS-Tween 20 containing 2% bovine serum albumin, and incubated for 1 h at room temperature with primary antibodies. Immunoreactive bands were revealed after a 1-h incubation with horseradish peroxidase-conjugated antirabbit antibodies and the signals were visualized by enhanced chemiluminescence.

### Migration and invasion assays

For migration assays, transwells (8- $\mu$ m pore size; Costar) were precoated with 0.15% gelatin in PBS by adding 600/100  $\mu$ L in the lower/upper chambers for 24 h at 4°C. The transwells were then washed with PBS and assembled into 24-well plates. For invasion assays, Matrigel (Becton Dickinson Labware; 0.5 mg/mL in PBS) was applied to each chamber and the plates were allowed to dry overnight in a laminar flow hood. In both migration and invasion assays, the upper chamber of each transwell was filled with 50  $\mu$ L of cells (1.0  $\times$  10<sup>6</sup> cells/mL) and pulmonary aortic smooth muscle cells were allowed to adhere for 30 min. The monolayers were then treated for 2 h by adding 50  $\mu$ L of 2-fold concentrated flavonoid solutions prepared in serum-free medium into the upper chamber and 600  $\mu$ L of the flavonoid solution (1 $\times$ ) into the lower chamber. After 2 h, PDGF-BB (10 ng/mL) was added to the lower chamber as a chemoattractant and the plate was placed at 37°C in 5% CO<sub>2</sub>/95% air for 3 h (migration) or 24 h (invasion). Cells that had



Flavonol	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Kaempferol (Kae)	H	H	OH
Myricetin (Myr)	OH	OH	OH
Flavone	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Luteolin (Lut)	OH	H	H
Apigenin (Api)	H	H	H

Fig 1. Chemical structures of flavones and flavonols used in this study.

migrated to the lower surface of the filters were fixed with 10% formalin phosphate and stained with 0.1% crystal violet in 20% methanol. The migration was quantitated using computer-assisted imaging (Northern Eclipse software, Empix Imaging) and data are expressed as the average density of migrated cells per four fields (magnification,  $\times 50$ ).

### Determination of VEGF by ELISA

Pulmonary aortic smooth muscle cells were exposed to either vehicle (0.1% DMSO) or flavonoid compounds (15  $\mu\text{mol/L}$ ) for 2 h before the addition of PDGF-BB (20 ng/mL in 1% fetal bovine serum). After a 24-h incubation, the amount of VEGF protein secreted into the conditioned medium was determined using the Quantikine Human VEGF immunoassay (R&D Systems) according to the manufacturer's protocol.

### Quantitative real-time reverse transcriptase-PCR

Confluent pulmonary aortic smooth muscle cells were incubated overnight in 1% serum and treated with vehicle or flavonoids (15  $\mu\text{mol/L}$ ) for 2 h. After incubation of the cells with PDGF-BB (20 ng/mL) for 30 or 60 min, cell monolayers were washed with PBS and total RNA was isolated using the QIAzol reagent (Qiagen) following the manufacturer's instructions. Ten micrograms of RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and primers annealing to the target VEGF sequence (forward, 5'-TGCTGTCTGGGTCATGG-3'; reverse, 5'-GGTGCAGCGTGGGACCACT-3') and 18S rRNA (forward, 5'-CGGCTACCACATCCAAGGAA; reverse 5'-GCTGGAAT-TACCGCGGCT-3') as the internal reference. PCR amplification of the cDNAs was done using a Bio-Rad IQ5 Multicolor Real-time PCR Detection System (Bio-Rad Laboratories Ltd.) with SYBR green fluorophore. After initial denaturation for 3 min, samples were subjected to 40 cycles of amplification (95°C for 10 s, annealing at 60°C for 15 s, and elongation at 72°C for 15 s) followed by a melt curve analysis in which the temperature was increased from 55°C to 91°C at a rate of 1°C/10 s. Protocols for each primer set were optimized using a series of five 2 $\times$  dilutions that were run on the same plate. All reactions were carried out in at least triplicate for each sample.

### In vivo Matrigel plug assay

The Matrigel plug assay was done essentially as described previously (26, 27). Briefly, female nude mice (CrI:CD-1-Foxn1<sup>tm</sup>; 20-25 g, 6 wk of age; Charles River Laboratories) were treated by injection s. c. into the ventral midline region of the right flank with 0.5 mL of phenol red-free Matrigel (BD Bioscience) containing apigenin (25  $\mu\text{mol/L}$ ) or luteolin (50  $\mu\text{mol/L}$ ), FGF-2 (250 ng/mL), VEGF (200 ng/mL), and heparin (0.0025 units/mL). Control mice were injected with Matrigel without FGF-2/VEGF. At the end of the study (day 12), some mice were injected through the tail vein with 200  $\mu\text{L}$  of high molecular weight (2,000,000 MW) FITC-labeled dextran (Sigma-Aldrich) and were sacrificed 10 min after injection. The Matrigel plugs were harvested, washed with PBS, and photographed. Matrigel plugs were either immediately frozen, lyophilized overnight, and processed for the determination of hemoglobin concentration, as described (27), or fixed in 10% formalin and examined by fluorescence microscopy (Zeiss-Axiovert 100). For histochemical analysis of formalin-fixed Matrigel samples, plugs were embedded in paraffin, cut into 5- $\mu\text{m}$  thickness, and then analyzed by H&E-safran staining.

### Statistical analysis

The data are presented as means  $\pm$  SE and statistical analyses were done with Student's *t* test when one group was compared with the control group.

## Results

### Inhibition of PDGF-BB-induced phosphorylation of PDGFR- $\beta$ by dietary flavones

We first examined PDGF-BB-induced phosphorylation of the PDGF receptor following a 18-hour preincubation of quiescent pulmonary aortic smooth muscle cells in the presence of kaempferol, luteolin, apigenin, or myricetin (15  $\mu\text{mol/L}$  each). Cells were stimulated with 50 ng/mL PDGF-BB for 5 minutes, and the extent of PDGFR- $\beta$  phosphorylation was assessed by immunoprecipitation of the receptor, followed by immunoblotting using a monoclonal antiphosphotyrosine antibody. Under these experimental conditions, we observed that luteolin and apigenin both dramatically reduced the phosphorylation of PDGFR- $\beta$  induced by PDGF-BB, whereas the tested flavonols (myricetin and kaempferol) had no inhibitory effect (Fig. 2A, *top*). Blotting of the membranes with an antibody directed against PDGFR- $\beta$  showed that the inhibition of PDGFR phosphorylation by apigenin and luteolin was not related to decreased receptor levels (Fig. 2A, *bottom*) nor to nonspecific cytotoxicity of the molecules toward pulmonary aortic smooth muscle cells (data not shown). The inhibition of PDGFR phosphorylation by the flavones likely involves the kinase activity of the receptor, as suggested by the reduction of immunolabeling following blotting of cell lysates with phosphospecific antibodies that recognize a major autophosphorylation site located within the kinase domain (Tyr<sup>857</sup>) of PDGFR (ref. 28; Fig. 2B). The inhibitory potency of apigenin and luteolin toward the PDGF receptor was very similar, with half-maximal inhibition being observed at concentrations of  $\sim 8$   $\mu\text{mol/L}$  for both flavones (Fig. 2C).

### Flavones inhibit PDGF-BB-mediated signaling pathways

We next investigated whether the inhibition of PDGFR- $\beta$  tyrosine phosphorylation by apigenin and luteolin also impairs the PDGF-BB-induced phosphorylation of ERK-1/2 and Akt, two key intracellular signaling events induced by this receptor (28). As shown in Fig. 3A (*top*), both apigenin and luteolin completely inhibited the phosphorylation of these two important signaling intermediates, whereas kaempferol and myricetin had no effect. This inhibitory effect was concentration dependent, with half-maximal inhibition occurring at concentrations very similar to those observed for the inhibitory effect of the molecules on PDGFR- $\beta$  (7  $\mu\text{mol/L}$ ; Fig. 3B). This thus suggests that the inhibitory effect of flavones on PDGFR- $\beta$  is likely to alter cellular events triggered by PDGF-BB.

### Flavones inhibit PDGF-induced pulmonary aortic smooth muscle cell migration and invasion

Numerous studies have shown that ERK-1/2 is important for PDGF-induced cell migration (29, 30), suggesting that the inhibition of PDGF-dependent ERK activation by apigenin and luteolin may interfere with this event. To test this possibility, cells were incubated for 2 hours with increasing concentrations of apigenin and luteolin and pulmonary aortic smooth muscle cell migration was induced by the addition of 10 ng/mL PDGF-BB. As shown in Fig. 4 (*left*), both apigenin and luteolin potently inhibited PDGF-BB-induced migration of pulmonary aortic smooth muscle cells, with a half-maximal

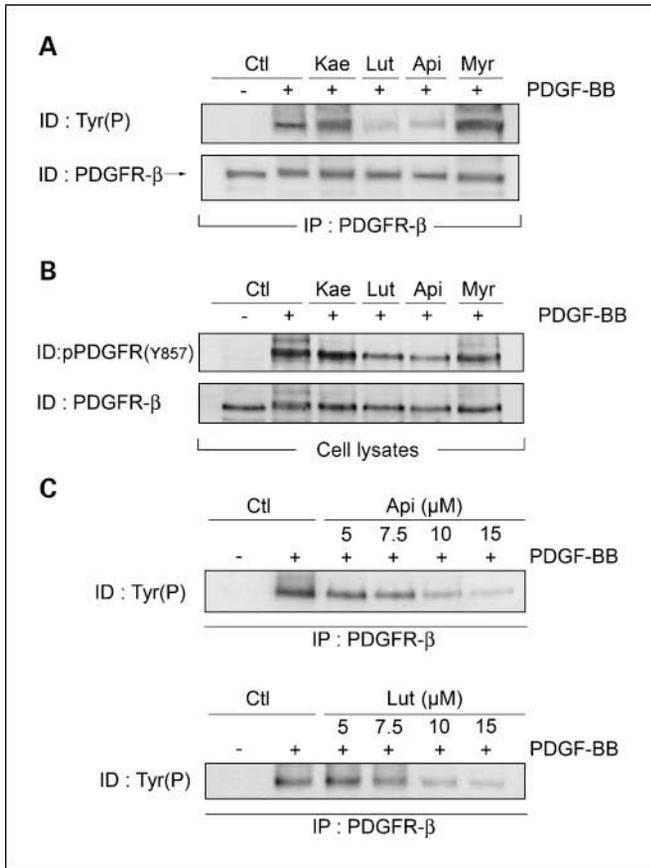


Fig. 2. Inhibition of PDGFR- $\beta$  phosphorylation by dietary flavonoids. *A*, pulmonary aortic smooth muscle cells were incubated for 18 h in the absence or presence of 15  $\mu$ mol/L kaempferol (*Kae*), luteolin (*Lut*), apigenin (*Api*), or myricetin (*Myr*) and the extent of PDGFR- $\beta$  tyrosine phosphorylation was monitored by immunoprecipitation and immunoblotting. *B*, pulmonary aortic smooth muscle cells were incubated as described in *A*, and phosphorylation of PDGFR- $\beta$  on Tyr<sup>857</sup> was monitored in whole-cell lysates using phosphospecific antibodies. *C*, pulmonary aortic smooth muscle cells were incubated with increasing concentrations of apigenin (*top*) or luteolin (*bottom*) and tyrosine phosphorylation levels of the PDGFR- $\beta$  were determined as described above. Representative of three independent experiments done on different cell preparations.

inhibitory effect occurring at  $\sim$ 2  $\mu$ mol/L for both molecules. Such a strong inhibitory effect of apigenin and luteolin was also observed for the PDGF-dependent invasion of a Matrigel matrix by pulmonary aortic smooth muscle cells (Fig. 3*A*, *right*). Interestingly, the inhibitory effects of apigenin and luteolin on pulmonary aortic smooth muscle cell migration were similar to that achieved by Gleevec, a drug used clinically for the treatment of chronic myeloid leukemia and whose mechanism of action includes inhibition of PDGFR- $\beta$  (Fig. 4).

**Flavones inhibit VEGF mRNA expression and VEGF secretion induced by PDGF-BB**

Production of PDGF by endothelial cells is required for the recruitment of smooth muscle cells (pericytes) and stabilization of tumor-associated neovessels (5). In turn, smooth muscle cells secrete VEGF, thereby creating a positive feedback loop that sustains angiogenesis (9). To determine whether the inhibitory effect of apigenin and luteolin on PDGFR phos-

phorylation and downstream signaling events impairs this event, pulmonary aortic smooth muscle cells were preincubated for 2 hours with the flavones and the expression of the VEGF mRNA was measured by real-time quantitative reverse transcriptase-PCR. As shown in Fig. 5, PDGF induced a rapid and transient increase in VEGF mRNA levels, a 4-fold stimulation being observed as early as 30 minutes after the addition of PDGF. Interestingly, pretreatment of the cells with apigenin completely abolished this increase in VEGF mRNA levels (Fig. 5) and abrogated the PDGF-dependent secretion of VEGF in the conditioned medium (Fig. 5). Luteolin was less effective in inhibiting these processes, with approximately 35% and 55% inhibition of VEGF mRNA expression and VEGF secretion by pulmonary aortic smooth muscle cells, respectively.

**Flavones inhibit FGF-2- and VEGF-induced angiogenesis *in vivo***

It was recently shown that the combination of FGF and VEGF synergistically induces neoangiogenesis *in vivo* through activation of PDGFR-mediated signaling (31), providing a useful assay to examine the inhibitory effect of fla-

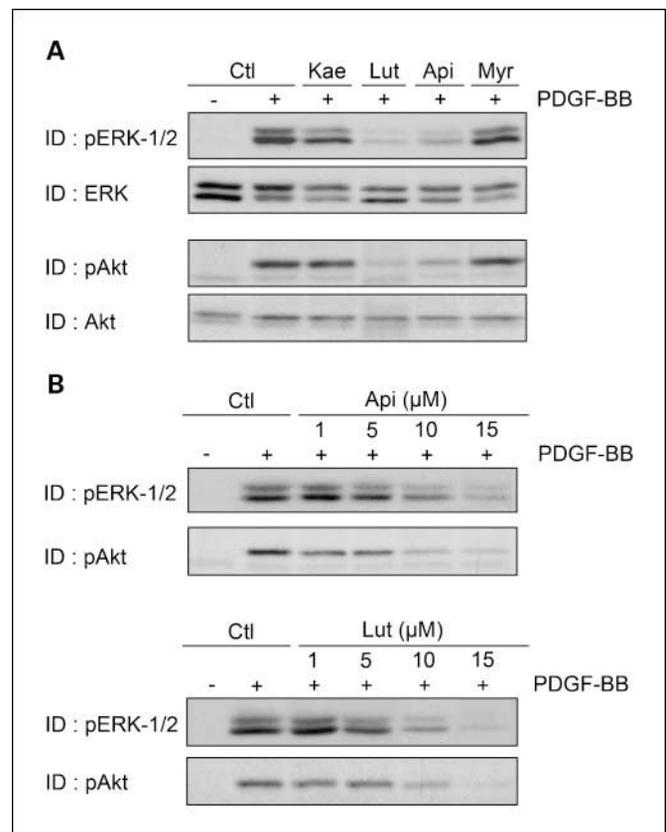


Fig. 3. Inhibition of PDGFR- $\beta$  downstream signaling events by flavones. *A*, pulmonary aortic smooth muscle cells were incubated for 18 h in the absence or presence of 15  $\mu$ mol/L kaempferol, luteolin, apigenin, or myricetin. Cells were lysed and equal amounts of protein were separated by SDS-PAGE. The phosphorylated forms of ERK-1/2 and Akt were visualized by immunoblotting with phosphospecific antibodies. *B*, pulmonary aortic smooth muscle cells were incubated with increasing concentrations of apigenin (*top*) or luteolin (*bottom*) and the phosphorylated forms of ERK-1/2 and Akt were visualized as described above. Representative of three independent experiments done on different cell preparations.

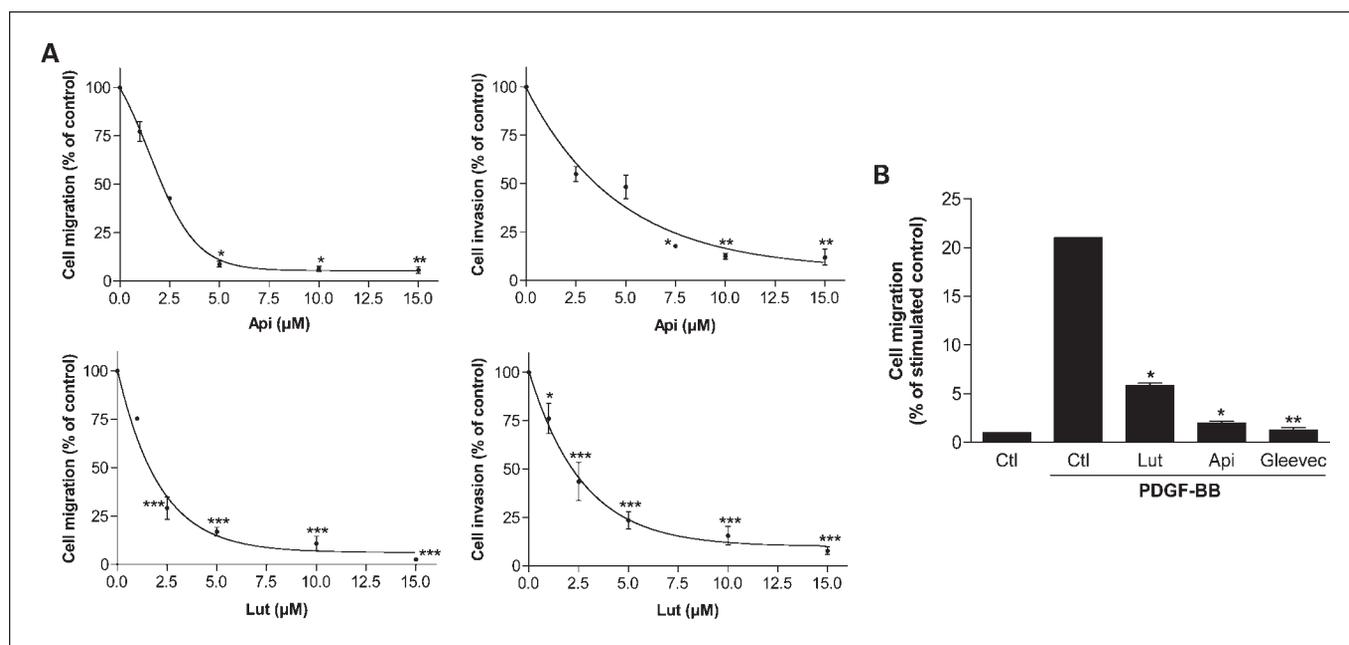


Fig. 4. Inhibition of PDGFR-dependent migration and invasion by flavones. *A*, pulmonary aortic smooth muscle cells were pretreated for 2 h with various concentrations of apigenin or luteolin and the extent of migration (*left*) or invasion (*right*) induced by PDGF was quantified as described in Materials and Methods. Points, mean of three independent experiments; bars, SE. \*\*\*,  $P < 0.001$ , versus PDGF-BB alone. *B*, pulmonary aortic smooth muscle cells were incubated for 18 h in the presence of 5  $\mu\text{mol/L}$  of luteolin, apigenin, or Gleevec and the extent of cell migration was determined as described in Materials and Methods. Columns, mean of three independent experiments; bars, SE.

flavones on PDGFR- $\beta$  activity on angiogenesis. Matrigel plugs that contained FGF-2 and VEGF with apigenin or luteolin were injected in mice and the extent of neovascularization was examined by fluorescence microscopy or immunohistochemistry. As shown in Fig. 6, whereas plugs containing FGF-2 and VEGF showed extensive vascularization with a well-defined branching structure indicative of a functioning capillary network, the addition of either apigenin (25  $\mu\text{mol/L}$ ) or luteolin (50  $\mu\text{mol/L}$ ) markedly reduced the formation of these vessels. However, at lower concentrations (25  $\mu\text{mol/L}$ ), luteolin had no significant inhibitory effect on neovascularization (data not shown). These results thus suggest that the inhibitory effect of dietary flavones on the PDGF-dependent signaling pathways may lead to impaired angiogenesis.

## Discussion

During angiogenesis, PDGF secreted by the endothelium plays a crucial role in the recruitment and integration of mural cells, such as pericytes and smooth muscle cells, in the vascular wall (5). In addition to inducing the stabilization of the newly formed vessels, these mural cells also contribute to effective neovascularization by serving as a primary source of VEGF, thereby promoting endothelial cell survival (9). The importance of mural cells in the function of tumor-associated blood vessels thus suggests that interference with PDGF-mediated signaling pathway may represent a promising molecular target for chemopreventive and therapeutic intervention (10).

In this study, we show that apigenin and luteolin, but not the structurally related flavonols kaempferol and myricetin,

inhibit PDGF-BB-dependent tyrosine phosphorylation of PDGFR- $\beta$  in smooth muscle cells and that this inhibitory effect is associated with an impairment of downstream signaling events triggered by this receptor, such as phosphorylation of the p42<sup>MAPK</sup> and p44<sup>MAPK</sup> forms (ERK-1/2) and of Akt. Importantly, the inhibition of these PDGFR-dependent event by both flavones was correlated with a marked reduction in the migratory and invasive properties of smooth muscle cells, two crucial events involved in the recruitment of these cells to neovessels. These inhibitory effects were observed following incubation of smooth muscle cells with low concentrations of the molecules, in keeping with our previous observations of the high sensitivity of PDGFR to molecules of dietary origins such as ellagic acid (32) and delphinidin (26). However, these results stand in contrast to those obtained by another group using rat smooth muscle cells, in which apigenin had no effect on PDGF-dependent activation of ERK (33) whereas kaempferol inhibited this process (34). Moreover, half-maximal inhibition of the phosphorylation of PDGFR, ERK, and Akt by luteolin was previously reported to occur at much higher concentrations in these cells (35). Whether these differences are related to a differential sensitivity of the receptor of rat and human origins to these molecules or to different experimental conditions remains to be determined. However, because the inhibitory effect of low concentrations of flavones on cell migration was similar to that of Gleevec, an anticancer agent whose mechanism of action includes inhibition of PDGFR- $\beta$  activity, our results strongly support the notion that blockade of PDGFR- $\beta$  is responsible for the inhibitory effect of apigenin and luteolin on smooth muscle cell migration. Although the mechanisms underlying the inhibition of PDGFR- $\beta$  activity by these

dietary-derived polyphenols remain to be established, we observed that the molecules significantly reduced phosphorylation of Tyr<sup>857</sup>, a residue that plays a role in the positive regulation of the kinase activity of the receptor (28). It is thus likely that both molecules directly interfere with the kinase activity PDGFR, in agreement with other studies showing that some flavonoids, such as epigallocatechin gallate from

green tea, inhibit the activation of receptor tyrosine kinases by competing with ATP for binding to the kinase domain of these receptors (36). However, because the inhibitory effect of luteolin and apigenin on the phosphorylation of the kinase domain of PDGFR was lower than that observed when total phosphorylation of the protein was monitored, the flavones are likely to interfere with additional regulatory components that modulate the phosphorylation of the receptor (28).

We also observed that stimulation of smooth muscle cells with PDGF induced a rapid up-regulation of VEGF mRNA levels, resulting in a marked (10-fold) increase of this cytokine in the culture medium. Interestingly, whereas both flavones interfered with these processes, this inhibitory effect was particularly important for apigenin (96% inhibition) whereas luteolin was less active (55% inhibition). Because these molecules inhibit PDGFR- $\beta$  activity to a similar extent, the stronger inhibitory effect of apigenin on VEGF expression indicates an interference of the molecule with additional pathways involved in this process. In this respect, hypoxia-inducible factor-1 $\alpha$  plays a central role in the transcription of the VEGF gene in cancer cells (37), and interference of apigenin with hypoxia-inducible factor-1 $\alpha$  function suppresses this up-regulation of VEGF expression (38–40). Because PDGF has previously been shown to induce hypoxia-inducible factor-1 $\alpha$  expression in smooth muscle cells (41), it is likely that an inhibitory effect of apigenin on this pathway may participate in its inhibition of VEGF expression by these cells.

Using an *in vivo* angiogenesis assay in which neovascularization in Matrigel plugs is induced by a combination of FGF-2 and VEGF, we found that relatively low concentrations of apigenin (25  $\mu$ mol/L) markedly reduced the formation of new vessels, whereas higher concentrations of luteolin were required to observe a similar inhibitory effect. Because previous studies have shown that angiogenesis triggered by FGF-2 and VEGF involves the activation of the PDGF-PDGFR- $\beta$  signaling axis (31), these results strongly suggest that the inhibitory effect of both flavones on PDGFR- $\beta$  receptor activity plays a major role in the observed reduction of neovascularization. Although both luteolin and apigenin were previously shown to inhibit endothelial cell proliferation (25, 42, 43), raising the possibility that this property may also contribute to impaired angiogenesis, it is noteworthy that luteolin was found to be at least 3- to 4-fold more potent than apigenin as an inhibitor of angiogenesis (25, 42). These observations stand in contrast to the 2-fold higher potency of apigenin as an inhibitor of neovascularization observed in our study and thus suggest that other mechanisms, such as the inhibition of VEGF production by smooth muscle cells, are involved in the inhibitory potential of these molecules on angiogenesis.

In summary, our results indicate that apigenin and luteolin interfere with PDGFR- $\beta$  activity and that this inhibitory effect is likely to have repercussions on angiogenesis by reducing smooth muscle cell migration, invasion, and VEGF expression by these cells. Although the bioavailability of dietary flavones seems to be relatively low (22), the inhibition of the PDGFR signaling axis reported here occurs at concentrations within an order of magnitude of the plasma concentration of apigenin measured after ingestion of 2 g parsley/kg of body weight (200 nmol/L; ref. 44). Because both apigenin and

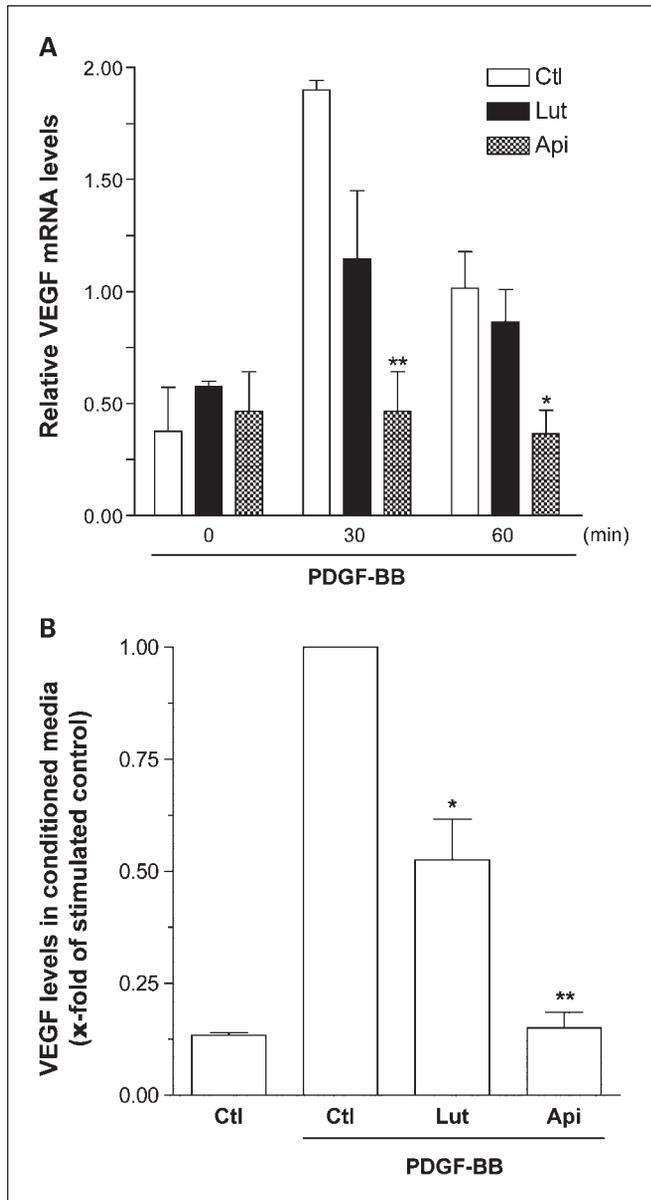


Fig. 5. Apigenin and luteolin inhibit PDGF-induced release of VEGF by smooth muscle cells. **A**, pulmonary aortic smooth muscle cells were incubated for 2 h in the absence or presence of apigenin or luteolin (15  $\mu$ mol/L each) and stimulated with PDGF (20 ng/mL) for the indicated periods of time. The expression of VEGF mRNA was measured by a real-time reverse transcriptase-PCR assay with SYBR green. *Columns*, mean of three independent experiments; *bars*, SE. **B**, pulmonary aortic smooth muscle cells were treated with apigenin and luteolin as described above and incubated in the presence of PDGF for 24 h. The amount of VEGF present in the conditioned media was determined by ELISA. *Columns*, mean of three independent experiments; *bars*, SE.

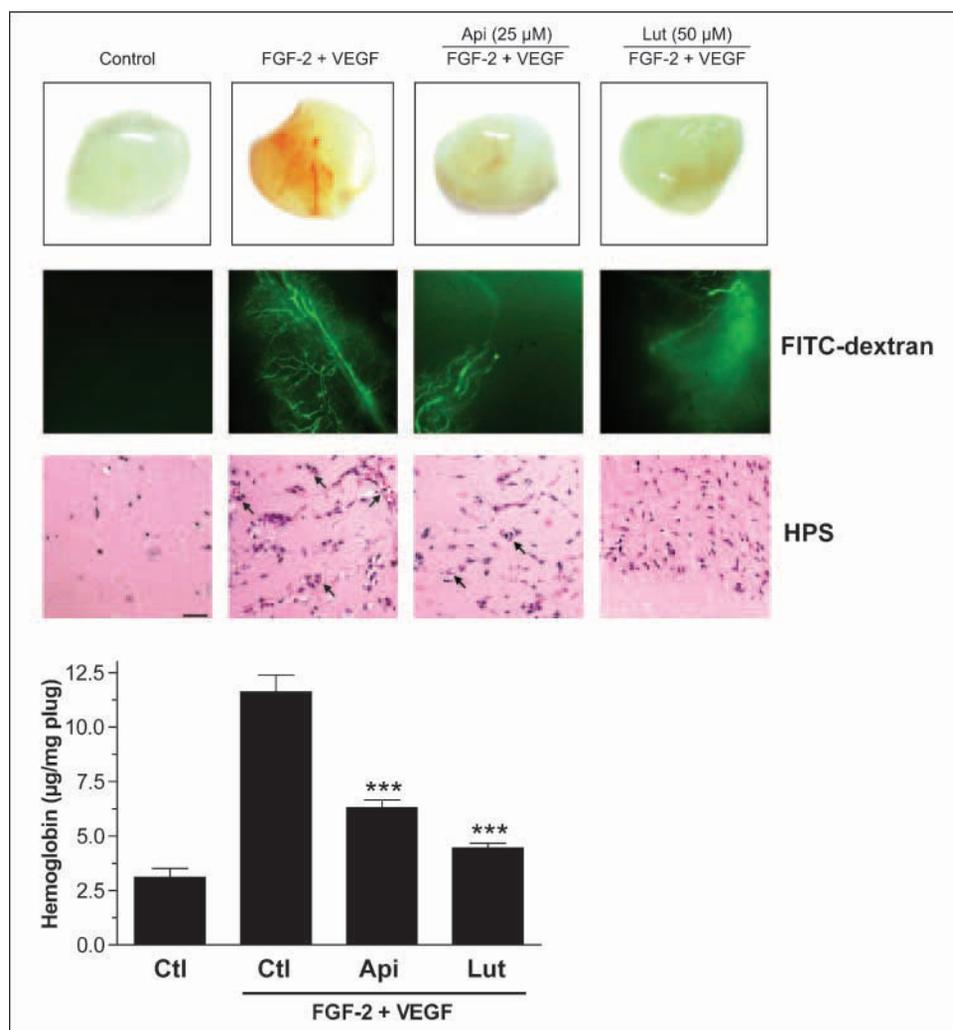


Fig. 6. Effect of apigenin and luteolin on synergistic angiogenesis *in vivo* induced by FGF-2 and VEGF. *Ctrl*: CD-1-Foxn1<sup>nu</sup> nude mice were injected s.c. with 0.5 mL of Matrigel containing FGF-2 (250 ng/mL), VEGF (200 ng/mL), and heparin (0.0025 units/mL) in the absence or in the presence of apigenin (25 µM) or luteolin (50 µM). After 12 d, Matrigel plugs were excised and analyzed as described in Materials and Methods. *Bar*, 50 µm.

luteolin exhibit slow pharmacokinetics that may enable accumulation of the molecules in tissues (45), it is thus tempting to speculate that the low concentrations of apigenin and luteolin that are shown here to inhibit PDGFR- $\beta$  are behaviorally achievable in humans and that the inclusion of foods rich in these molecules in the diet, such as celery and parsley, may have chemopreventive effects through the inhibition of angiogenesis.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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