Apigenin decreases expression of the myofibroblast phenotype

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Abstract We investigated the effect of the dietary flavonoid apigenin on myofibroblast function. We report that in myofibroblasts treated with apigenin, proliferation and basal levels of α1(I) collagen and α-smooth muscle actin mRNAs were markedly reduced. Apigenin also attenuated the transforming growth factor-β-stimulated increases of α1(I) collagen and α-smooth muscle actin mRNAs. Characterization of the apigenin effects indicates that apigenin reduces both the stability of the α1(I) collagen mRNA and the rate of transcription of the α1(I) collagen gene through a cycloheximide-sensitive pathway. Western blot analyses indicate that Akt activity is reduced in apigenin-treated myofibroblasts.

Key words: α1(I) Collagen; mRNA stability; Apigenin; Flavonoid; α-Smooth muscle actin; Myofibroblast; IMR-90

1. Introduction

Flavonoids are diphenyl propanoids found in edible plants and are categorized as flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids. Flavonoids display anti-proliferative and anti-inflammatory properties [1–3]. For example, quercetin (3,3′,4′,5,7-pentahydroflavone) has been reported to relieve pain, lower body temperature, and inhibit viral uncoating [4]. The activities of enzyme systems involved in the initiation and maintenance of the inflammatory and immune responses are modulated by flavonoids. These include serine and threonine protein kinases, phospholipases, lipoxygenase, and cyclooxygenase [4–8]. Flavonoids are also potent inhibitors of processes involved in mitogen signaling and DNA synthesis, including mitogen activation of receptor tyrosine kinases [9,10], protein kinase C [11,12] and topoisomerase II [13]. Flavopiridol, a novel semisynthetic flavone analogue of rohitukinean, is currently undergoing phase II trials for the treatment of a variety of malignancies [14,15].

Apigenin (4′,5,7-trihydroxyflavone), a common dietary flavonoid, is a non-toxic and non-mutagenic flavone. Apigenin affects a variety of cellular processes related to cell proliferation, including inhibition of mitogen-activated protein (MAP) kinase [16] and phosphoinositol-3 kinase (PI3K). Apigenin markedly inhibits the migration of endothelial cells and capillary formation in vitro, independently of its inhibition of hyaluronidase activity [17]. As an inhibitor of cell proliferation, apigenin may play a role in the prevention of carcinogenesis. Apigenin at concentrations of 1–80 μM induces growth inhibition and cell loss in cultures of colonic tumor cells [18]. Moreover, apigenin suppresses 12-*O*-tetradecanoylphorbol-13-acetate-mediated tumor promotion of mouse skin [19]. Apigenin has been shown to induce cell cycle arrest in both epidermal cells and fibroblasts and to inhibit skin tumorigenesis in murine models [19]. Intraperitoneal administration of apigenin at the time of injection of B16-BL6 cells into syngeneic mice, results in a significant, dose-dependent delay of tumor growth, without toxicity [20].

In fibrotic diseases, deposition of extracellular matrix is modulated by increases in cell numbers as well as by regulation of transcription, translation and post-translational modifications. The anti-proliferative and anti-inflammatory effects of apigenin suggest that apigenin may modulate processes involved in fibrogenesis. Specifically, apigenin may modulate myofibroblast functions. We found that in human lung myofibroblasts, apigenin blocked thymidine incorporation and reduced basal and transforming growth factor-β (TGF-β)-stimulated levels of α-smooth muscle actin mRNA and α1(I) collagen mRNA.

2. Materials and methods

2.1. Materials

Apigenin (Calbiochem) and LY294002 (Calbiochem) were reconstituted at 5 mg/ml in dimethyl sulfoxide (DMSO). [^{3}H]thymidine was purchased from NEN DuPont (Boston, MA, USA). Recombinant porcine TGF-β was purchased from R&D Systems (Minneapolis, MN, USA). Anti-AKT antibodies (New England Biolabs), anti-phospho-serine^{473}-AKT antibodies (New England Biolabs), anti-phospho-Erk1/2 antibodies (Upstate Biotechnology), and anti-Erk1/2 antibodies (Upstate Biotechnology) were used according to manufacturer's instructions.

2.2. Tissue culture

Human embryonic lung fibroblasts (IMR-90, Institute for Medical Research, Camden, NJ, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.37 g sodium bicarbonate/100 ml, 10% (v/v) fetal bovine serum (FBS), 100 U penicillin/ml, 10 μg streptomycin/ml, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. After confluence, the serum content of the medium was reduced to 0.4% FBS. Experimentation was performed with fibroblasts that had undergone less than 35 population doublings and presented no evidence of senescence by light microscopy.

2.3. Nuclear run-on assay

Confluent, quiescent fibroblasts were washed twice with Puck's saline and scraped into a lysis buffer (10 mM Tris–HCl pH 7.4, 10 mM

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NaCl, 3 mM $MgCl_2$, 0.5% NP-40). Following two low-speed spins, the pellet was reconstituted in glycerol buffer (50 mM Tris–HCl pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). In vitro labeling of nascent RNA and hybridization with cDNA immobilized on nitrocellulose filters was performed according to the methods reported previously [21,22].

2.4. Western blotting

PAGE was performed under reducing conditions using 10% polyacrylamide gels as described [23]. Samples (100 μg) for SDS–PAGE and Western blotting were prepared from the cell layer of quiescent confluent fibroblasts grown in 100-mm tissue culture dishes. The cell layer was dissolved in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride, and 600 nM okadaic acid in phosphate-buffered saline (PBS) and centrifuged ($12\,000 \times g$ for 20 min) at 4°C. Protein concentrations were determined by Bradford assay. Gels were transferred to nitrocellulose filters (Schleicher and Schuell) and blocked according to manufacturer's specifications [23].

2.5. Luciferase assay

IMR-90 myofibroblasts were plated in 6-well plates (350 000/well) in DMEM supplemented with 10% FBS. After 24 h, the cultures were washed with PBS and incubated with the 3TP-LUX reporter (1 μg/well) mixed with Lipofectamine Plus according to manufacturer's instructions (Gibco Life Technologies, Rockville, MD, USA) for 3 h. The transfection medium was replaced with DMEM supplemented with 10% FBS without antibiotics and the cultures were incubated for an additional 20 h. The cultures were incubated in 0.4% FBS without antibiotics for 16 h before experimentation.

3. Results

Our focus was to determine the effect of apigenin on the maintenance of the myofibroblast phenotype. As an in vitro model, we used primary human lung fibroblasts (IMR-90) that constitutively express both type I collagen and α-smooth muscle actin, thus characterizing them as myofibroblasts [24]. We determined the effect of apigenin on cellular proliferation. The cultures were treated with varying doses of apigenin for 24 h, then assayed for thymidine incorporation as previously described [25]. We found that apigenin blocked thymidine incorporation in a dose-dependent manner, with 200 nM apigenin having no effect and 20 μM apigenin inducing a maximal effect (Fig. 1). We found that concentrations of apigenin higher than 30 μM with a final concentration of 0.1% DMSO formed a precipitate when added to the tissue culture medium.

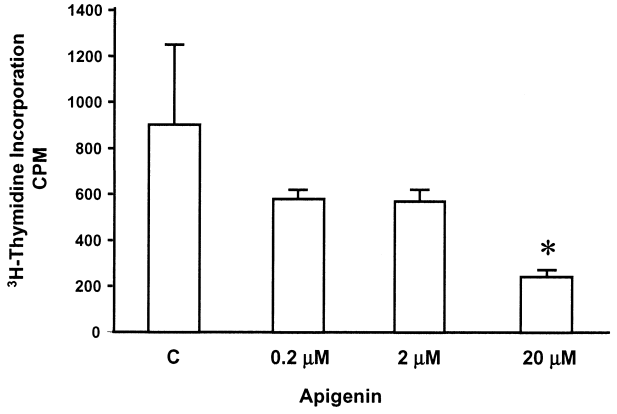

Fig. 1. Dose-dependent inhibition of cell proliferation by apigenin. Subconfluent myofibroblasts in 24-well plates were treated with the indicated concentrations of apigenin for 24 h. The cultures were pulse-labeled with [^{3}H]thymidine (0.5 Ci/well), washed and scintillation counted. Values are mean CPM+SE, $n=3$. Results are representative of two independent experiments. *$P<0.05$ compared with untreated cultures (C).

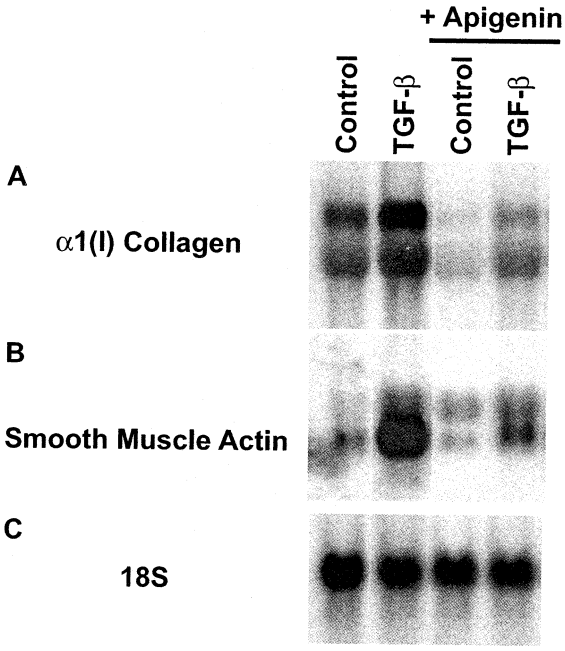

Fig. 2. Effect of apigenin on α1(I) collagen and α-smooth muscle actin mRNA levels. Confluent quiescent myofibroblasts were treated with 20 μM apigenin for 24 h and/or stimulated with TGF-β (1 ng/ml) as indicated. Total RNA was isolated, resolved electrophoretically (10 μg), and Northern blotted with probes for (A) α1(I) collagen mRNA (B) α-smooth muscle actin mRNA or (C) 18S rRNA as described. Results are representative of three independent experiments.

To investigate the effect of apigenin on the myofibroblast phenotype, we assessed the effect of apigenin on steady-state levels of α1(I) collagen mRNA and α-smooth muscle actin mRNA in both quiescent and growth factor-stimulated myofibroblasts. Northern blot analyses indicate that quiescent myofibroblasts constitutively express low levels of α1(I) collagen mRNA. Stimulation with TGF-β induced an increase of α1(I) collagen mRNA steady-state levels. Following treatment with 20 μM apigenin for 24 h, the basal level of α1(I) collagen mRNA was reduced and the TGF-β-stimulated increase of α1(I) collagen mRNA was attenuated (Fig. 2a). A similar pattern of expression was observed with α-smooth muscle actin mRNA. Quiescent myofibroblasts constitutively express α-smooth muscle actin and stimulation with TGF-β induced an increase of steady-state levels. As observed with α1(I) collagen mRNA, treatment with apigenin decreased basal levels of α-smooth muscle actin mRNA and attenuated the TGF-β-stimulated increase of α-smooth muscle actin mRNA (Fig. 2b). In contrast, we found that apigenin did not alter basal or TGF-β-stimulated levels of connective tissue growth factor (CTGF) mRNA (data not shown).

The decrease of basal α1(I) collagen mRNA by apigenin is time-dependent (Fig. 3). Quiescent myofibroblasts were incubated with 20 μM apigenin for 8, 16 and 24 h. Following 8 h apigenin treatment, the basal levels of α1(I) collagen mRNA remained unchanged. A noticeable decrease of α1(I) collagen mRNA was observed following 16 h of apigenin treatment. A maximal decrease of α1(I) collagen mRNA was observed at 24 h following apigenin treatment. Similarly, the apigenin

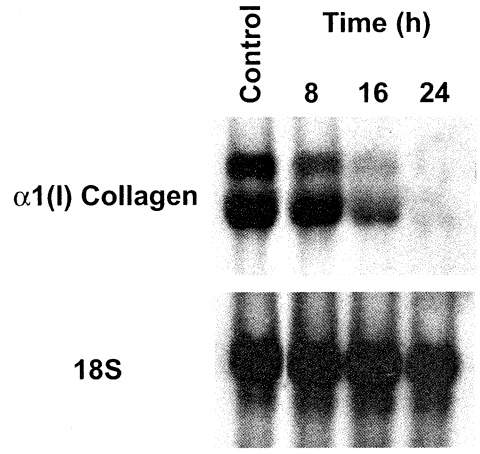

Fig. 3. Kinetic studies following apigenin treatment. Confluent, quiescent myofibroblasts were treated with apigenin (20 μM) for the indicated times in hours. Total RNA was isolated, resolved electrophoretically (10 μg), and Northern blotted with probes for α1(I) collagen mRNA or 18S rRNA as described. Results are representative of two independent experiments.

response was dose-dependent with a maximal decrease of α1(I) collagen mRNA achieved at 20 μM apigenin (data not shown).

The effect of apigenin on α1(I) collagen mRNA levels was further characterized by inhibiting protein synthesis with cycloheximide. The addition of 5 μM cycloheximide did not affect basal α1(I) collagen mRNA levels. In myofibroblasts treated with cycloheximide, apigenin failed to induce a reduction of basal α1(I) collagen mRNA levels (Fig. 4).

To assess the stability of the α1(I) collagen mRNA, transcription was blocked with actinomycin D. Without ongoing transcription, α1(I) collagen mRNA decays with a half-life of 8–12 h [26]. Myofibroblasts were treated with apigenin for 6 h

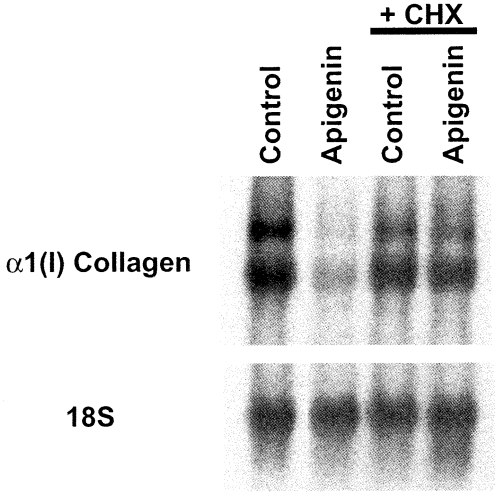

Fig. 4. Effect of cycloheximide on apigenin-induced decreases of α1(I) collagen mRNA. Confluent, quiescent myofibroblasts were treated with 5 μM cycloheximide (CHX) and apigenin (20 μM) for 24 h as indicated. Total RNA was isolated, resolved electrophoretically (10 μg), and Northern blotted with probes for α1(I) collagen mRNA or 18S rRNA as described.

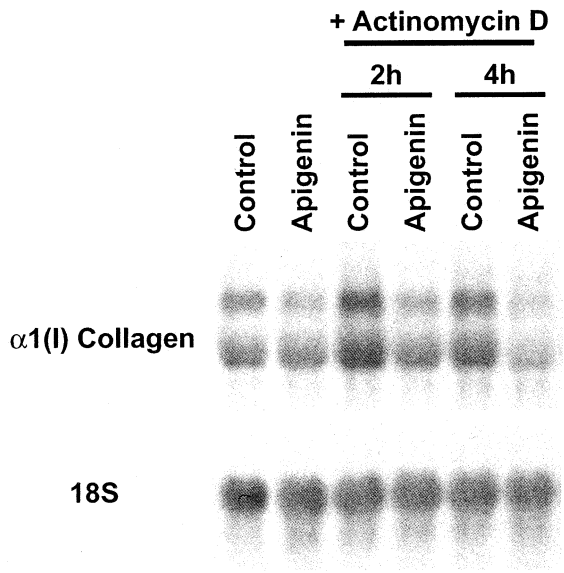

Fig. 5. Effect of actinomycin D on the apigenin-induced steady-state levels of α1(I) collagen mRNA. Confluent, quiescent myofibroblasts were untreated or pre-treated with apigenin (20 μM) for 6 h. Actinomycin D (5 μM) was added to the indicated cultures. Total RNA was isolated at the indicated times, resolved electrophoretically (10 μg), and Northern blotted with probes for α1(I) collagen mRNA or 18S rRNA as described. Results are representative of three independent experiments.

before transcription was blocked with actinomycin D. In myofibroblasts incubated with the combination of apigenin and actinomycin D, the rate of decline in the level of α1(I) collagen mRNA was greater compared to myofibroblasts that were incubated with only actinomycin D (Fig. 5). These results suggest that apigenin reduced the stability of the message. The blots were stripped and re-probed for expression of α-smooth muscle actin mRNA. We found that apigenin decreased the stability of the α-smooth muscle actin mRNA (data not shown).

The effect of apigenin on the rate of transcription was assessed by nuclear run-on assays. Nuclei were isolated from untreated and apigenin-treated myofibroblasts. Basal levels of α1(I) collagen transcription were not affected by apigenin. TGF-β induced an increase in α1(I) collagen transcription, consistent with previous reports [26]. However, treatment with apigenin inhibited this TGF-β-induced increase in the rate of transcription of the α1(I) collagen gene. In addition, apigenin did not affect transcription of glyceraldehyde 3-phosphate dehydrogenase (Fig. 6). No hybridization was observed with filters containing plasmids without inserts (data not shown).

To further investigate transcriptional activation, we used the luciferase reporter construct p3TP-LUX that was engineered to be responsive to TGF-β [27]. In myofibroblasts transfected with p3TP-LUX, TGF-β stimulates an increase in luciferase activity. However, in apigenin-treated myofibroblasts TGF-β failed to stimulate the reporter construct (Fig. 7). These results along with the insensitivity of the TGF-β-stimulated transcription of CTGF to apigenin demonstrate that apigenin interferes with TGF-β-stimulated transcription in a gene-specific manner.

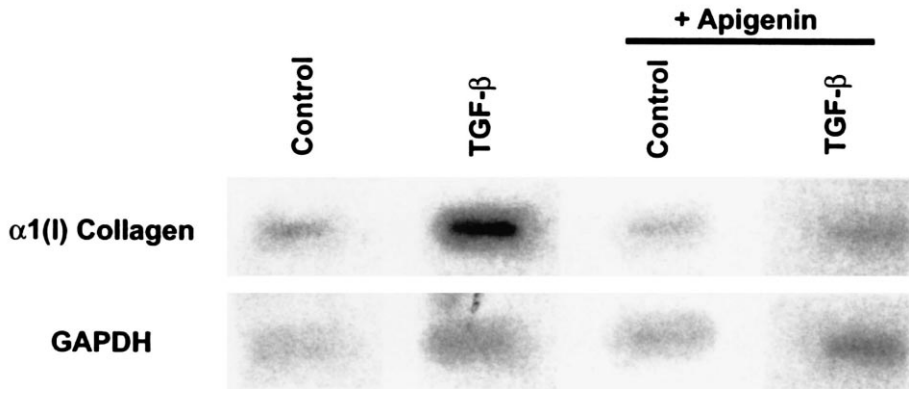

Fig. 6. Nuclear run-on assay for α1(I) collagen. Confluent, quiescent myofibroblasts were pre-treated with 20 μM apigenin for 30 min, then stimulated with TGF-β (1 ng/ml) as indicated for 16 h. Nuclei were harvested and the levels of transcription for α1(I) collagen and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes were assayed as described.

Apigenin is a reported inhibitor of MAP kinase Erk1/2 [16]. To determine if apigenin regulated α1(I) collagen levels via this pathway, we assessed the effect of apigenin on the levels of activated Erk1/2 in both quiescent and growth factor-stimulated myofibroblasts with Western blot analyses with antibodies that detect phosphorylated Erk1/2. We did not detect an increase in Erk1/2 activity at 10 min or at 16 h following TGF-β stimulation. In addition, apigenin did not induce a decrease in the basal levels of active Erk1/2. We found that insulin stimulated an increase of Erk1/2 activity, but apigenin induced only a modest attenuation. The blots were stripped and re-probed with an antibody that detects total Erk1/2. Treatment with apigenin did not alter expression of Erk1/2 (Fig. 8).

The actinomycin studies (Fig. 5) indicate that apigenin reduces the stability of the α1(I) collagen mRNA. We previously reported a decrease of α1(I) collagen mRNA stability in myofibroblasts treated with the PI3K inhibitors wortmannin or LY294002[28]. Because the steady-state levels of α-smooth muscle actin mRNA paralleled the steady-state levels of α1(I) collagen mRNA, we investigated the relationship between PI3K and the steady-state levels of α-smooth muscle actin mRNA. To block PI3K activity, myofibroblasts were treated with LY294002. Northern blot analyses indicate that basal levels and TGF-β-stimulated levels of α-smooth muscle actin mRNA were decreased in LY294002-treated myofibroblasts (Fig. 9).

The data indicate that apigenin attenuated expression of α1(I) collagen mRNA in part through decreased stability of the message. We hypothesized that the change in steady-state levels of α-smooth muscle actin mRNA may also be due to decreased stability of the message and that the change in stability may be mediated through decreased PI3K activity. As a surrogate for PI3K activity, we monitored the levels of active Akt by Western blot analyses with antibodies that detect phospho-serine^{473}-Akt. In quiescent myofibroblasts, there is a small amount of active Akt. Following treatment with apigenin, the amount of active Akt is reduced. To demonstrate that apigenin blocks growth factor-stimulated activation of Akt, myofibroblasts were stimulated with insulin, a growth factor that is known to activate PI3K and Akt. However, in apigenin-treated myofibroblasts, insulin failed to acti-

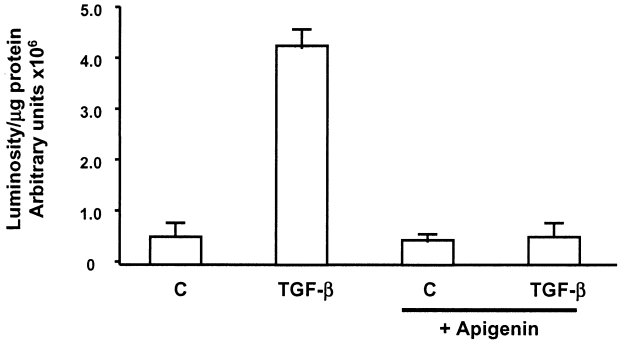

Fig. 7. Apigenin effect on TGF-β-stimulated luciferase activity. Myofibroblasts were transiently transfected with the p3TP-LUX reporter construct in 6-well plates (1 μg/well) using Lipofectamine Plus according to manufacturer's instructions. Myofibroblasts were untreated (C) or incubated with 20 μM apigenin for 10 min, then stimulated with 1 ng/ml TGF-β as indicated. Luciferase activity/μg protein was determined after 6 h. Values are means of triplicates+ standard deviation, representative of four independent experiments.

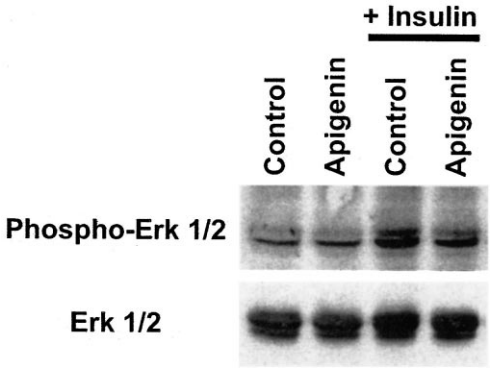

Fig. 8. Phospho-Erk1/2 and total Erk1/2 levels following treatment with apigenin. Confluent, quiescent myofibroblasts were treated with apigenin at 20 μM and/or insulin (2 μg/ml) for 16 h as indicated. The cell layer was harvested and 100 μg total protein was resolved by PAGE as described. Blots were probed with an anti-phospho-Erk1/2 antibody. Blots were stripped and re-probed with anti-Erk1/2 antibody. Results are representative of two independent experiments.

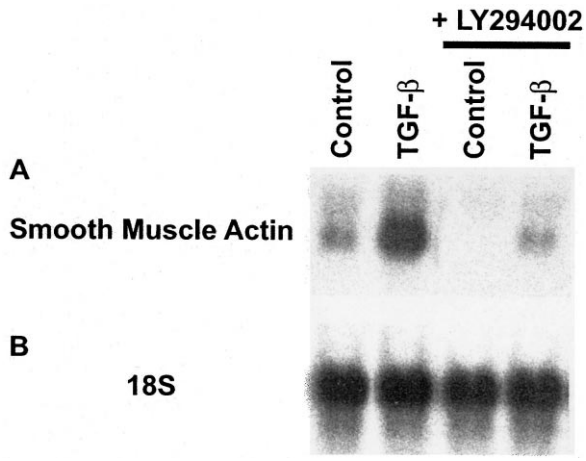

Fig. 9. Effect of LY294002 on the TGF-β-stimulated increase of α-smooth muscle actin mRNA. Confluent, quiescent myofibroblasts were pre-treated with LY294002 (20 μM), and after 10 min, TGF-β (1 ng/ml) was added to the cultures for 16 h as indicated. Total RNA was isolated, resolved electrophoretically (10 μg), and Northern blotted with probes for α-smooth muscle actin or 18S rRNA as described. Results are representative of two independent experiments.

vate Akt. The blots were stripped and re-probed with anti-Akt antibody, demonstrating that the total amount of Akt was not decreased by treatment with apigenin (Fig. 10).

4. Discussion

TGF-β plays a key role in the development of fibrotic tissues by inducing increased expression of matrix proteins, including type I collagen. Depending on the cell type, TGF-β stimulates an increase of α1(I) collagen mRNA levels by increasing transcription of the α1(I) collagen gene and by increasing the stability of the message [29]. The results of the run-on assay (Fig. 6) clearly indicate that the TGF-β-stimulated transcription of the α1(I) collagen gene is blocked by apigenin. In addition, TGF-β did not stimulate transcription from the p3TP-LUX reporter in apigenin-treated myofibroblasts. In contrast, the TGF-β-stimulated transcription of CTGF is unaffected in apigenin-treated myofibroblasts. Thus, it appears that apigenin disrupts TGF-β-stimulated transcription in a gene-specific manner. Apigenin does not appear to disrupt signaling of receptor-activated SMAD3 since both CTGF and p3TP-LUX reporter transcription requires SMAD3 [30–32]. Apigenin may block the activity of a co-transcription factor such as AP-1. AP-1 binds SMAD3 and modulates SMAD3-stimulated promoter activity in a number of genes [33–35]. The transcription of 3TP-LUX is activated by SMAD3 and AP-1 [27]. Depending on the cell type and the stimulus, the transcription of the α1(I) collagen gene is activated or repressed by members of the AP-1 family of transcription factors [36–41].

We found that apigenin largely inhibited the TGF-β-stimulated induction of α1(I) collagen mRNA by blocking transcription of the α1(I) collagen gene. However, TGF-β did stimulate a modest increase of α1(I) collagen mRNA in apigenin-treated myofibroblasts. This residual activity may be due to apigenin-insensitive mechanisms activated by TGF-β. Alternatively, it is possible that the concentration of apigenin, which was dictated by the limited solubility of apigenin in aqueous solution, does not completely inactivate the mechanisms that regulate mRNA stability.

Apigenin is known to inhibit PI3K [42]. Western analyses confirmed that apigenin inhibited this pathway, as determined by decreased phospho-serine^{473}-AKT levels. We previously reported that inhibition of PI3K by LY294002 or wortmannin decreased α1(I) collagen mRNA levels through a mechanism that involves reduced mRNA stability without changes in the rate of transcription [28]. In the studies presented here, apigenin induced a decrease in α1(I) collagen mRNA stability and a reduction of the levels of activated Akt, suggesting that apigenin functions at least in part to decrease basal α1(I) collagen mRNA levels through this mechanism. Apigenin also blocked the TGF-β-induced increase in the rate of transcription, indicating another regulatory mechanism. It appears that TGF-β stimulates transcription of α1(I) collagen through a PI3K-independent mechanism.

Apigenin is described as an inhibitor of MAP kinase, but the available evidence supporting this function is not strong [16]. Alterations in MAP kinase activity were reported by others to influence α1(I) collagen mRNA levels either positively or negatively depending on the cell type [43,44]. We did detect a modest decrease of activated Erk1/2 activity in human lung myofibroblasts treated with apigenin. Moreover, we found that basal and TGF-β-stimulated levels of α1(I) collagen mRNA were not affected by PD98059, the MAP kinase kinase inhibitor (data not shown).

In the normal lung, interstitial fibroblasts do not express detectable levels of α-smooth muscle actin. Following lung injury, fibroblasts are activated and express cytoplasmic microfilament bundles containing α-smooth muscle actin. These myofibroblasts increase cell volume and produce large quantities of type I collagen [45,46]. The differentiation of the

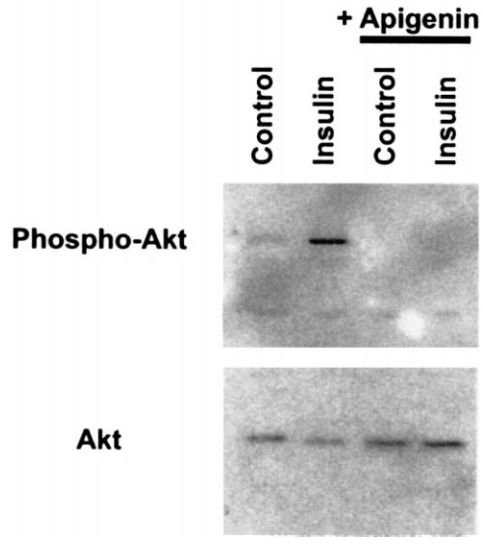

Fig. 10. Phospho-serine^{473}-AKT and total AKT levels following treatment with apigenin. Confluent, quiescent myofibroblasts were treated with apigenin (20 μM) and/or insulin (2 μg/ml) for 16 h as indicated. The cell layer was harvested and 100 μg total protein was resolved by PAGE as described. Blots were probed with an anti-phospho-serine^{473}-AKT antibody. Blots were stripped and re-probed with anti-AKT antibody. Results are representative of two independent experiments.

myofibroblast in the kidney, liver and other tissues is associated with the onset of fibrogenesis [47–49]. TGF-β appears to be important in regulating the myofibroblast phenotype [49]. In the studies presented, the myofibroblasts constitutively express low levels of α-smooth muscle actin mRNA and α1(I) collagen mRNA. We find that apigenin down-regulates basal α-smooth muscle actin and α1(I) collagen mRNA expression, demonstrating that apigenin interfered with the appearance of the myofibroblast phenotype. Because the stability of α1(I) collagen mRNA correlates with PI3K-Akt activity, we propose that maintenance of the myofibroblast phenotype is dependent on persistent PI3K activation.

Apigenin is a common dietary flavonoid that, unlike the flavonoids quercetin and kaempferol, is non-toxic and non-mutagenic [50,51]. Flavonoids are widely distributed throughout the plant kingdom, as such they comprise a significant component of the human diet. The average intake of the flavonols, quercetin, myricetin and kaempferol, and the flavones, luteolin and apigenin, in The Netherlands is 23 mg/day [52]. Dietary flavonoids were considered non-absorbable because they are bound to sugars as β-glycosides. However, human absorption of the quercetin glycosides from onions is twice that of the pure aglycone [53]. Flavonoid intake from a normal diet is estimated to be 1 g/day, resulting in blood concentrations of 1–2 μM [54]. Apigenin is a relatively abundant flavonoid present in fruits and vegetables such as parsley, cherries, grapes, onions, and broccoli. The concentration of apigenin used in our experiments is slightly greater than the level of flavonoids found in blood in humans on a normal diet. This suggests the possibility of in vivo activity from flavonoids consumed in normal diets. In this study, we have identified a novel molecular action of apigenin, that is, the down-regulation of α1(I) collagen and α-smooth muscle mRNAs. We suggest that dietary or supplemental consumption of apigenin may modulate myofibroblast activity in vivo.

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