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Effect of celecoxib on proliferation, collagen expression, ERK1/2 and SMAD2/3 phosphorylation in NIH/3T3 fibroblasts

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ABSTRACT

In the present study, the effects of celecoxib on proliferation, collagen expression, ERK1/2 and SMAD2/3 phosphorylation in NIH/3T3 fibroblasts were investigated. NIH/3T3 fibroblasts stimulated with fibroblast growth factor-2 (FGF-2) or transforming growth factor-β1 (TGF-β1) were examined in the presence of celecoxib. Proliferation was assessed by MTT assays; ERK1/2 expression and SMAD2/3 expression were assessed by quantitative RT-PCR and western blotting; ERK1/2 phosphorylation and SMAD2/3 phosphorylation were assessed by western blot analysis. The results indicated that celecoxib could suppress cell proliferation stimulated by FGF-2 (IC_{50} FGF + group, 75 ± 1.9 μmol/l) and TGF-β1 (IC_{50} TGF + group, 48 ± 1.4 μmol/l), by inhibiting ERK1/2 phosphorylation but not ERK1/2 expression. Celecoxib also suppressed collagen expression (0.35-fold COL3 and 0.43-fold COL1 at 320 μmol/l celecoxib relative to the untreated control after stimulation with TGF-β1 for 3 h, $P<0.01$), by inhibiting SMAD2/3 phosphorylation but not SMAD2/3 expression. The suppression of NIH/3T3 fibroblast proliferation and collagen expression upon stimulation by FGF-2 and TGF-β1 is likely a result of the inhibition of ERK1/2 and SMAD2/3 phosphorylation by celecoxib.

1. Introduction

Celecoxib is a non-steroidal anti-inflammatory drug (NSAID) that was developed as a selective inhibitor of cyclooxygenase-2 (COX-2) and approved by the U.S. Food and Drug Administration for the treatment of various forms of arthritis and the management of acute and chronic pain (Frampton et al., 2007). In addition, it was more recently approved as an oral adjunct to prevent colon cancer development in patients with familial adenomatous polyposis and is presently being investigated for its chemotherapeutic potential in the therapy of advanced cancers (Futagami et al., 2007; Gore, 2004; Half et al., 2009). Celecoxib was also found to inhibit hepatic and cardiac fibrosis (Chávez et al., 2010; Paik et al., 2009; Wang et al., 2010). Furthermore, celecoxib was reported to retard all types of wound healing, and reduce scar and adhesion formation (Baatar et al., 2002; Berenguer et al., 2002; De Hingh et al., 2006; Greene et al., 2005; Wilgus et al., 2003), which are all processes of tissue fibrosis.

Celecoxib is unique among the coxibs and traditional NSAIDs. COX-2 inhibition was considered to play an important role in the pharmacologic effects of NSAIDs. However, in laboratory studies it was discovered that celecoxib was able to suppress tumour growth in the absence of any apparent involvement of COX-2, and some additional pharmacologic activities associated with this drug were found (Schönthal, 2007). Furthermore, other studies showed that celecoxib exerted its apoptosis-induction activity through COX-2-independent mechanisms (Paik et al., 2009). Celecoxib's pharmacologic activities and corresponding mechanisms remain very unclear, and maybe depend on the target cell type and the specific conditions. In the present study, we sought to investigate the effect of celecoxib on proliferation, collagen expression, ERK1/2 and SMAD2/3 phosphorylation in NIH/3T3 fibroblasts, and to delineate the mechanism by which celecoxib is implicated in retarding all types of wound healing, and reducing scar and adhesion formation.

2. Materials and methods

2.1. Cell culture and stimulation

NIH/3T3 cells, which were purchased from Sigma-Aldrich (St. Louis, MO), were maintained in Dulbecco's modified Eagle medium (Gibco, Langley) supplemented with 10% calf serum (Gibco, Langley), 2 mM L-glutamine, penicillin, and streptomycin at 37 °C in a 5% CO_2 incubator. The cells were seeded onto 90 mm tissue culture dishes and grown to 80% confluence, then used for ERK1/2 (Cell Signaling, Beverly, MA), SMAD2/3 (Cell Signaling, Beverly, MA), collagen (Santa Cruz, CA), and cell proliferation assays.

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2.2. Measurement of cell proliferation by methylthiazole tetrazolium (MTT) assay

Approximately 5×10^4/ml NIH/3T3 cells were seeded in 96-well culture plates. We treated stimulated and unstimulated NIH/3T3 cells by FGF-2 with increasing concentrations of celecoxib, which was first dissolved in dimethyl sulfoxide (DMSO), and was then added in medium. After 48 h, these cells were harvested, washed, and incubated with 10 μl of MTT solution for 4 h. Subsequently, 100 μl of DMSO was added to dissolve the crystals. The plates were incubated at room temperature for 10 min before measurement of the absorbance at 570 nm using a microplate reader (Bio-Rad Laboratories, Veenendaal, The Netherlands).. Each sample was assayed in triplicate and each experiment was repeated three times.

2.3. Quantitative RT-PCR

Total RNA was isolated from NIH/3T3 cells using the TRIzol reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Isolated RNA was converted into cDNAs using Reverse Transcriptase M-MLV (TaKaRa, Japan). Quantitative RT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen) and a Detection System (Cepheid, CA). Quantitative RT-PCR was performed in a total reaction volume of 25 μl containing 12.5 μl of Quanti-Tect SYBR Green RT-PCR master mix, 3 μl of cDNA template and 0.2 μmol of each target-specific primer designed to amplify a part of each gene. Specific sense and antisense primers used are shown in Table 1. After PCR, a melting curve analysis was performed to demonstrate the specificity of each PCR product as a single peak. A control reaction containing all the components except for the template was included in all experiments. COL3 and COL1 gene expression levels were normalized to the corresponding gene expression levels of mouse β-actin.

2.4. Western blot

NIH/3T3 cells were harvested and lysed with lysis buffer. The cell lysates were centrifuged at 13,000 *g* for 15 min at 4 °C, and the supernatants were collected for western blot analysis. Equal amounts of protein were separated by SDS–PAGE using a 12% gel and electrotransferred to nitrocellulose membranes (Millipore, CA). After blocking with 5% non-fat milk, the membranes were incubated with antibodies against COL3, COL1 (Santa Cruz, CA), ERK1/2 and SMAD2/3 (Cell Signaling, Beverly, MA) for 1 h at room temperature. A monoclonal anti-GAPDH antibody (Cell Signaling, Beverly, MA) was used as a control. After washing, the membranes were incubated with horseradish peroxidase-conjugated IgG (Acris Antibodies, Herford, Germany) for 1 h, and immunoreactive bands were detected by chemiluminescence (Amersham).

Table 1
PCR Primers of target genes.

Target genes	PCR primers
ERK2	Sense: 5′-GGCGGGCCCGGAGATGGTC-3′ Antisense: 5′-TGAAGCGCAGTAGGATTTTTAT-3′
COL3	Sense: 5′-GGCGCCCCAGGAGAAAAG-3′ Antisense: 5′-CAGGAGGACCAGGGCGACCAC-3′
COL1	Sense: 5′-GACCTCCGGCTCCTGCTCCTCTTA-3′ Antisense: 5′-ACAGCACTCGCCCTCCCGTTTTTG-3′
β-actin	Sense: 5′-AGAGGGAAATCGTGCGTGAC-3′ Antisense: 5′-CCATACCCAGGAAGGAAGGCT-3′

2.5. Statistical analysis

Data are shown as the mean ± standard deviation (S.D.). Statistical analysis was carried out by one way analysis of variance (ANOVA) with $P<0.05$ (*) and $P<0.01$ (**) representing significance. Dose–response curves were fitted using nonlinear regression analysis and IC_{50}s of inhibitors were calculated. Values of $P<0.05$ were considered statistically significant. These statistical analyses were done using SigmaPlot and Graphic Pad software.

3. Results

3.1. Celecoxib inhibits NIH/3T3 fibroblast proliferation stimulated by FGF-2 and TGF-β1

As shown in Fig. 1A, MTT revealed that FGF-2 (1.27-fold, $P<0.05$) and TGF-β1 (1.297-fold, $P<0.05$) significantly promoted NIH/3T3 fibroblast proliferation. But the proliferations were markedly inhibited by celecoxib treatment (IC_{50} FGF + group, 75 ± 1.9 μmol/l) (IC_{50} TGF + group, 48 ± 1.4 μmol/l) (Fig. 1B). The effects of celecoxib were dose-dependent, with greater inhibition at higher doses.

3.2. Celecoxib inhibits up-regulated p-ERK1/2 stimulated by FGF-2 in NIH/3T3 fibroblasts

As shown in Fig. 2, FGF-2 significantly increased p-ERK1/2 in NIH/3T3 fibroblasts (2.9-fold and 4.9-fold after 15 min and 30 min, respectively, $P<0.01$). But this activation was markedly inhibited by celecoxib treatment (0.3-fold with 320 μmol/l celecoxib and 0.57-fold with 40 μmol/l celecoxib relative to the no celecoxib group after FGF-2 stimulation for 30 min, $P<0.01$). Interestingly, the basal

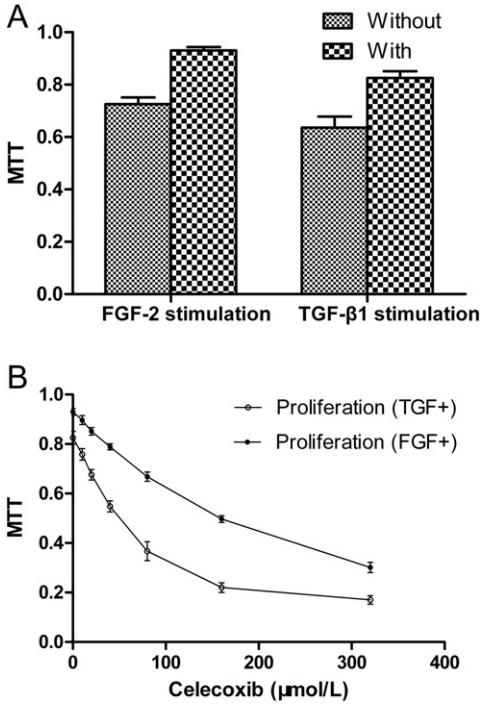

Fig. 1. Cell proliferation was assessed by MTT after 48 h at different concentrations of celecoxib (0–320 μmol/l) with FGF-2 stimulation (FGF + group) or with TGF-β1 stimulation (TGF + group). The FGF + group was treated daily with 5 ng/ml FGF-2, and the TGF + group was treated daily with 1 ng/ml TGF-β1. The results were obtained from three independent experiments and the MTT results are expressed as the mean ± S.D.

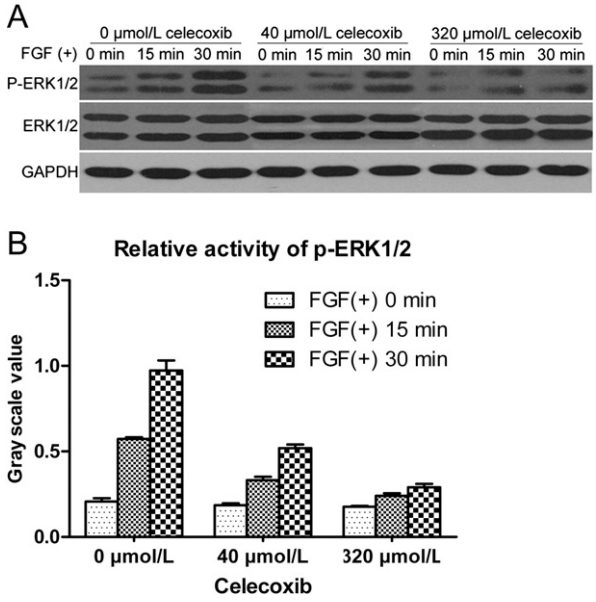

Fig. 2. NIH/3T3 cells were stimulated with 5 ng/ml FGF-2 after serum starvation for 20 h and celecoxib treatment for 1 h. (A) Western blot analyses were performed at the different concentrations of celecoxib. The results were obtained from three independent experiments, (B) Densitometric of the blots is analysed, and the relative activity of p-ERK1/2(p-ERK1/2/ERK1/2) is expressed as the mean ± S.D.

p-ERK1/2 level was not affected by celecoxib. In addition, neither FGF-2 nor celecoxib affected ERK1/2 expression.

3.3. Celecoxib inhibits up-regulated p-ERK1/2 stimulated by TGF-β1 in NIH/3T3 fibroblasts

As shown in Fig. 3, TGF-β1 significantly increased p-ERK1/2 in NIH/3T3 fibroblasts (3.8-fold and 7.9-fold after 15 min and 30 min, respectively, $P<0.01$). This activation was markedly inhibited by celecoxib treatment (0.25-fold with 320 μmol/l celecoxib and 0.54-fold with 40 μmol/l celecoxib relative to the no celecoxib group after TGF-β1 stimulation for 30 min, $P<0.01$). Interestingly, the basal p-ERK1/2 level was not affected by celecoxib. In addition, neither TGF-β1 nor celecoxib affected ERK1/2 expression.

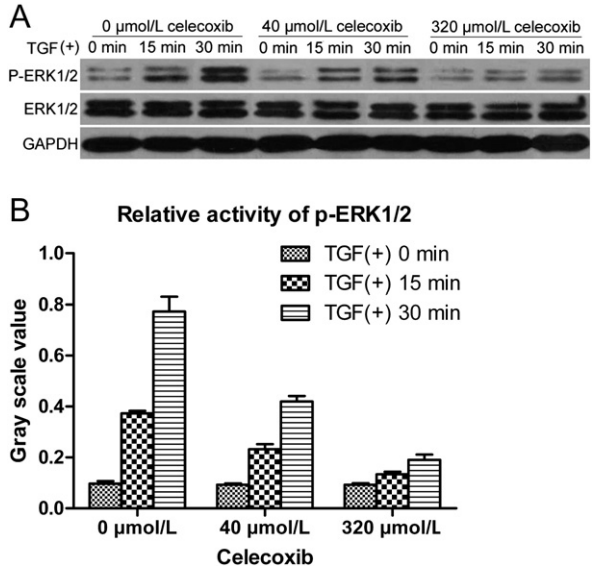

Fig. 3. NIH/3T3 cells were stimulated with 1 ng/ml TGF-β1 after serum starvation for 20 h and celecoxib treatment for 1 h. (A) Western blot analyses were performed at the different concentrations of celecoxib. The results were obtained from three independent experiments. (B) Densitometric of the blots is analysed, and the relative activity of p-ERK1/2(p-ERK1/2/ERK1/2) is expressed as the mean ± S.D.

3.4. Celecoxib inhibits collagen expression of NIH/3T3 fibroblasts stimulated by TGF-β1

As shown in Fig. 4, TGF-β1 significantly increased COL3 and COL1 expression in NIH/3T3 fibroblasts (3.55-fold COL3 and 4.18-fold COL3 after 1.5 h and 3 h, respectively, $P<0.01$) (1.2-fold COL1 and 3.08-fold COL1 after 1.5 h and 3 h, respectively, $P<0.01$). This promotion was markedly inhibited by celecoxib treatment (0.35-fold COL3 with 320 μmol/l celecoxib and 0.72-fold COL3 with 40 μmol/l celecoxib relative to the no celecoxib group after TGF-β1 stimulation for 3 h, $P<0.01$) (0.43-fold COL1 with 320 μmol/l celecoxib and 0.74-fold COL1 with 40 μmol/l celecoxib relative to the no celecoxib group after TGF-β1 stimulation for 30 min, $P<0.01$). Celecoxib treatment was not able to inhibit basal COL3 and COL1 expression.

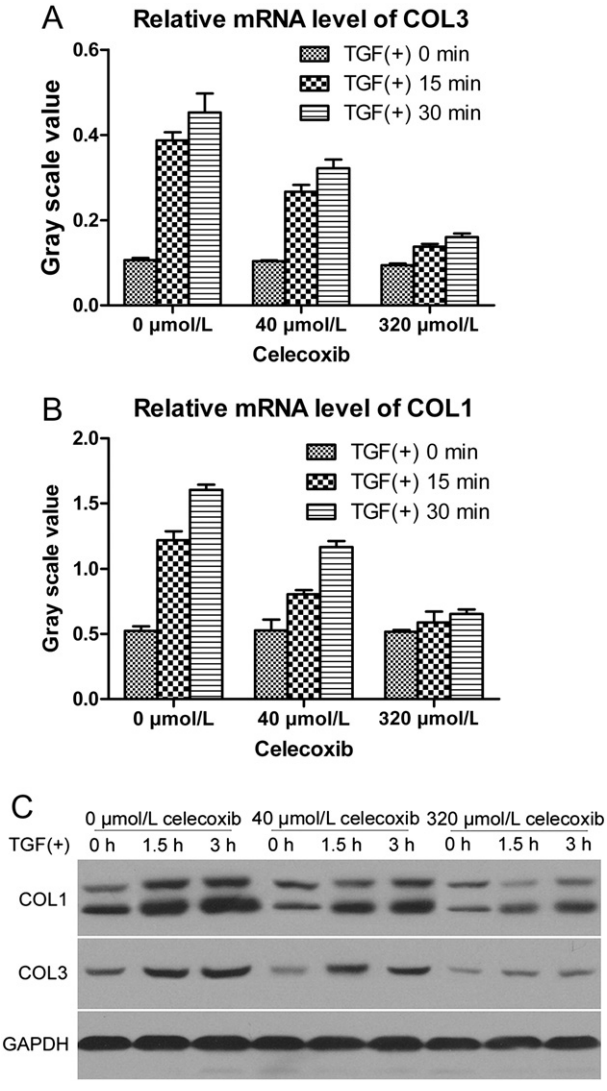

Fig. 4. NIH/3T3 cells were stimulated with 1 ng/ml TGF-β1 after serum starvation for 20 h and celecoxib treatment for 1 h. (A, B) Quantitative RT-PCR and (C) Western blot analyses were performed at the different concentrations of celecoxib. The results were obtained from three independent experiments, and the relative COL3 and COL1 mRNA levels (COL3/GAPDH and COL1/GAPDH) are expressed as the mean ± S.D.

3.5. Celecoxib inhibits up-regulated p-SMAD2/3 stimulated by TGF-β1 in NIH/3T3 fibroblasts

As shown in Fig. 5, TGF-β1 significantly increased p-SMAD2/3 in NIH/3T3 fibroblasts (1.93-fold and 3-fold after 15 min and 30 min, respectively, $P<0.01$). This activation was markedly inhibited by celecoxib treatment (0.42-fold with 320 μmol/l celecoxib and 0.63-fold with 40 μmol/l celecoxib relative to the no celecoxib group after TGF-β1 stimulation for 30 min, $P<0.01$). Interestingly, SMAD2/3 expression of NIH/3T3 fibroblasts was not affected by TGF-β1 and celecoxib treatment. In addition, basal p-SMAD2/3 levels were not affected by celecoxib.

4. Discussion

Celecoxib is presently being investigated for its chemotherapeutic potential in the therapy of advanced cancers. For example, celecoxib may prevent colon cancer development in patients with familial adenomatous polyposis. Recently celecoxib has been demonstrated to inhibit viability and induce apoptosis in some cellular systems such as the human glioma cell line U251 and HeLa cell line (Fukada et al., 2007; Tuynman et al., 2005; Zhou et al., 2010). These mechanisms are correlated with the effects of celecoxib on its inhibition of cancer. Furthermore, some studies showed that celecoxib exerted its apoptosis-induction and viability inhibition activities through COX-2-independent mechanisms, which may be PI3K/Akt-dependent, and survivin and bcl-2-related (Lev-Ari et al., 2007; Liu et al., 2008; Pang et al., 2007). Cell proliferation and collagen production also play very important roles in cancer formation and tissue fibrosis. These processes depend on the MAPK and SMAD pathways, in which ERK1/2 and SMAD2/3 is supposed to be crucial. However, the effects of celecoxib on proliferation, collagen expression, ERK1/2 and SMAD2/3 phosphorylation in NIH/3T3 fibroblast were unclear.

The results of the present study supported the hypothesis that, apart from being a selective COX-2 inhibitor, celecoxib is also an inhibitor of ERK1/2 and SMAD2/3 phosphorylation, suppressing NIH/3T3 fibroblast proliferation and collagen expression. Celecoxib has different roles in ERK1/2 activation in different cells. For example, in human non-small cell lung cancer cells, treatment with celecoxib alone had no effect (Ko et al., 2009). However, in human hepatic stellate cells (HSCs), celecoxib significantly attenuated ERK1/2 activation. In addition, celecoxib upregulated p-ERK1/2 in COX-2-negative human ovarian cancer cells, but not in COX-2-positive human ovarian cancer cells (Bijman et al., 2008). In NIH/3T3 fibroblasts, our present study demonstrated that celecoxib could inhibit up-regulated p-ERK1/2 stimulated by FGF-2 and TGF-β1. Our present study also showed that the growth of NIH/3T3 fibroblasts was inhibited by celecoxib after stimulation with FGF-2 and TGF-β1. It was known that celecoxib could inhibit the proliferation of all types of cells, such as endometrial stromal cells, retinal pigment epithelial cells, and oral squamous cancer cells (Amrite et al., 2008; Kong et al., 2008; Kwak et al., 2007). In view of the key role of ERK1/2 in mitogen-activated cell proliferation, celecoxib was considered to reduce NIH/3T3 fibroblast proliferation brought about by stimulation with FGF-2 and TGF-β1 by inhibiting up-regulation of p-ERK1/2. Celecoxib also exerted a different effect on collagen expression in depending on the type of cells. For example, celecoxib inhibited COL1 mRNA and protein production in HSCs [Paik et al., 2009], but the expression of COL1 and COL3 remained unchanged in tendon cells treated with celecoxib (Tsai et al., 2007). As in HSCs, celecoxib also inhibited COL1 and COL3 protein production in NIH/3T3 fibroblasts in the present study. Interestingly, this study also demonstrated that celecoxib could inhibit up-regulated p-SMAD2/3 stimulated by TGF-β1 in NIH/3T3 fibroblasts, which contribute to collagen expression. Thus celecoxib may inhibit COL1 and COL3 protein production in NIH/3T3 fibroblasts by inhibiting up-regulated p-SMAD2/3. A recent study strongly indicated that one of the ERK1/2 isoforms, ERK2, plays a synergistic role in SMAD2/3-mediated collagen synthesis initiated by TGF-β1 in NIH/3T3 cells (Li et al., 2009a, 2009b). Previously, we also demonstrated that siRNA-mediated ablation of ERK2 could markedly inhibit COL1 and COL3 synthesis stimulated by TGF-β1 in rat adhesion tissue fibroblasts (Li et al., 2009a, 2009b). In the present study, celecoxib could inhibit up-regulated p-ERK1/2 stimulated by TGF-β1. Thus inhibition of stimulated collagen synthesis may also be partly a result of the effect of celecoxib in inhibiting p-ERK1/2 stimulated by TGF-β1. However, celecoxib had little effect on the basal levels of p-ERK1/2 and p-SMAD2/3, and thus, had no influence on normal cells, which would account, at least in part, for its low cytotoxicity.

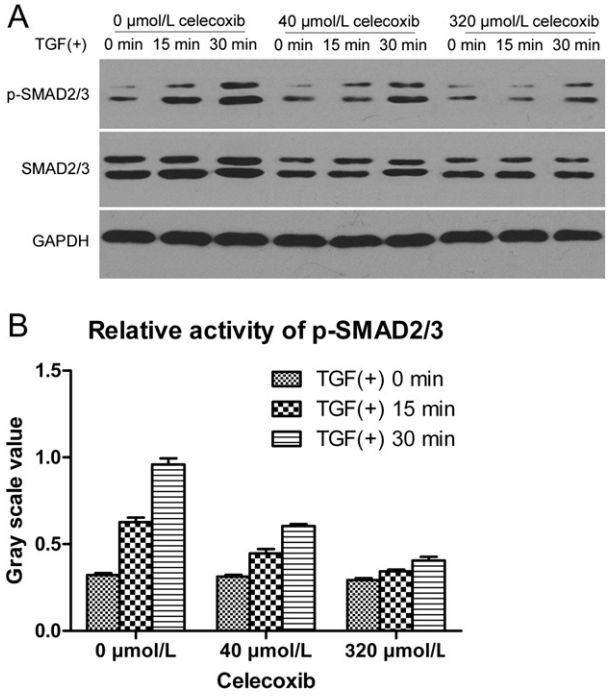

Fig. 5. NIH/3T3 cells were stimulated with 1 ng/ml TGF-β1 after serum starvation for 20 h and celecoxib treatment for 1 h. (A) Western blot analyses were performed at the different concentrations of celecoxib. The results were obtained from three independent experiments. (B) Densitometric of the blots is analysed, and the relative activity of p-SMAD2/3(p-SMAD2/3/SMAD2/3) is expressed as the mean ± S.D.

Fibrosis is a complex tissue disease whose predominant characteristics are the excessive and abnormal deposition of extracellular matrix (ECM) components (J. Uitto et al., 2000; F. Verrecchia et al., 2004), that may affect various organs, including joint, tendon, lung, liver, cardiovascular, kidney, and skin. TGF-β1 plays a very important role in collagen expression (Fine et al., 1990). This processes lead to the formation of fibrosis through the SMAD pathway, in which SMAD2/3 is supposed to be crucial. In addition, FGF-2 and TGF-β1 are known to have a strong mitogenic activity on NIH3T3 fibroblasts, which leads to cell proliferation and fibrosis formation through ERK1/2 activation (Benzakour et al., 1992). In view of the effects of celecoxib on cell proliferation, ERK1/2 phosphorylation, collagen expression and SMAD2/3 phosphorylation in NIH/3T3 fibroblasts, this study should prove useful for the exploration of celecoxib's effects on some fibrosis processes correlated with fibroblasts, such as wound healing, scar and adhesion formation. In addition, with regard to cell proliferation and collagen synthesis, even ECM synthesis, the mesenchymal cells have similar effects as celecoxib. Thus our results may also shed some light on other fibrotic diseases.

Acknowledgments

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