

# p38 MAPK mediates the regulation of $\alpha 1(I)$ procollagen mRNA levels by TNF- $\alpha$ and TGF- $\beta$ in a cell line of rat hepatic stellate cells<sup>1</sup>

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**Abstract** The role of members of the mitogen-activated protein kinase (MAPK) family on tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )-mediated down-regulation of *colla1* gene was studied. TNF- $\alpha$  increased extracellular-regulated kinase and Jun-N-terminal kinase phosphorylation, but these effects were not related to its inhibitory effect on  $\alpha 1(I)$  procollagen (*colla1*) mRNA levels. Phosphorylation of p38 MAPK was decreased in response to TNF- $\alpha$ , and the specific p38 MAPK inhibitor SB203580 mimicked the effect of TNF- $\alpha$  on *colla1* mRNA levels. Transforming growth factor  $\beta$  (TGF- $\beta$ ) increased p38 MAPK phosphorylation and SB203580 prevented the induction of *colla1* mRNA levels by TGF- $\beta$ . These results suggest that p38 MAPK plays an important role in regulating the expression of *colla1* in hepatic stellate cells in response to cytokines. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** p38 MAPK; TNF- $\alpha$ ; TGF- $\beta$ ; Hepatic stellate cells; A-SMase;  $\alpha 1(I)$  procollagen

## 1. Introduction

Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) is one of the main cytokines involved in regulating proliferation and extracellular matrix production by hepatic stellate cells (HSC), the cell type responsible for collagen accumulation in the liver [1–3]. TNF- $\alpha$  down-regulates the expression of  $\alpha 1(I)$  and  $\alpha 2(I)$  procollagen mRNAs in cultured HSC due to transcriptional inhibition of both genes [4,5]. The molecular mechanisms by which TNF- $\alpha$  decreases the expression of collagen in HSC have not been fully elucidated. It has been suggested that a

G protein could be involved in the inhibition of  $\alpha 1(I)$  procollagen (*colla1*) expression, and that sphingomyelin and ceramide are two of the intracellular mediators of TNF- $\alpha$  [6].

Sphingomyelin breakdown by sphingomyelinases and the consequent ceramide release have been demonstrated to mediate cell proliferation, differentiation and apoptosis in different cell types [7,8]. The diverse outcomes that result from ceramide accumulation could be caused by the different subcellular locations of sphingomyelin hydrolysis. There are two main types of sphingomyelinases, the neutral sphingomyelinase (N-SMase), and the acidic sphingomyelinase (A-SMase), each isoform leading to ceramide formation within plasmalemmal and endosomal membranes, respectively [9]. Activation of SMases leads to phosphorylation and activation of different members of the mitogen-activated protein kinase (MAPK) family. The N-SMase activity has been related to the activation of the extracellular-regulated kinase (ERK) cascade, a signaling pathway involved in increased collagen production by HSC and osteoclastic cells [10,11]. On the other hand, A-SMase triggers the activation of the stress-activated cascade, leading finally to the activation of kinases like Jun-N-terminal kinase (JNK) and p38 MAPK [12,13]. JNK activation has also been shown to be responsible for increased collagen production induced by UV light in HSC [14]. However, until now the relationship between MAPK activation by TNF- $\alpha$  and the down-regulation of collagen production elicited by this cytokine in HSC remained to be investigated.

In this report we studied the involvement of ERK, JNK and p38 MAPK in the TNF- $\alpha$ -mediated down-regulation of *colla1* mRNA levels in HSC. Our results indicate that p38 MAPK is a key mediator of the anti-fibrogenic effect exerted by TNF- $\alpha$  in HSC. The role played by p38 MAPK on the regulation of *colla1* gene was further demonstrated using transforming growth factor  $\beta$  (TGF- $\beta$ ) as an inductor of collagen expression in HSC.

## 2. Materials and methods

### 2.1. Reagents

TNF- $\alpha$  and TGF- $\beta$  were from Roche diagnostics (Barcelona, Spain). Cell culture reagents were from Gibco BRL (Grand Island, NY, USA). SB203580, SB202474 and PD98059 were purchased from Calbiochem® (Germany). L-JNK11 was from ALEXIS® Biochemicals (Lausen, Switzerland). SR33557 was provided by Dr. Jaffrèzou.

### 2.2. Cell culture and treatments

All the experiments were performed using the HSC line CFSC-2G

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**Abbreviations:** TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; TGF- $\beta$ , transforming growth factor  $\beta$ ; HSC, hepatic stellate cells; *colla1*,  $\alpha 1(I)$  procollagen; ERK, extracellular-regulated kinase; JNK, Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; A-SMase, acidic sphingomyelinase; N-SMase, neutral sphingomyelinase

[15]. Cells were cultured in minimum essential medium (MEM) supplemented with 10% bovine fetal serum and non-essential amino acids for 36 h, after which the medium was replaced for a serum-free MEM. Treatments were carried out 12 h later.

Unless otherwise indicated, HSC were treated with either 10 ng/ml TNF- $\alpha$  or 8 ng/ml TGF- $\beta$  for 24 h, these concentrations and time of treatment had been established in previous works [4,16]. In some experiments cells were pretreated for 30 min with either 0.1 mM SR33557, 10  $\mu$ M SB203580 and SB202474, 10  $\mu$ M PD98059 or 10  $\mu$ M L-JNK11. The specificity of these inhibitors at concentrations used in the present study has been demonstrated in previous reports [17–19].

### 2.3. Determination of oligonucleosomal (histone-associated) DNA fragments

The presence of soluble histone–DNA complexes was measured by the Cell Death Detection assay (Boehringer, Mannheim, Germany). For this assay, HSC were seeded on 24-well plates at a density of 80 000 cells/well. Cell death enzyme-linked immunosorbent assays were performed according to the manufacturer's instructions. Specific enrichment of mono- and oligonucleosomes released into the cytoplasm (enrichment factor, EF) was calculated as the ratio between the absorbance values of the samples obtained from treated and control cells.

### 2.4. RNA extraction and RT-PCR

Total RNA was extracted by the method of Chomczynski and Sacchi [20]. Reverse transcription polymerase chain reaction (RT-PCR) was performed using Platinum PCR Supermix kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Total RNA (0.75  $\mu$ g) was reverse-transcribed in a reaction volume of 20  $\mu$ l by using 1 U/ $\mu$ l murine leukemia virus reverse transcriptase at 42°C. PCR was performed by using the following primers: sense 5'-CTCTGGGATGATGGCTTGAG-3' and antisense 5'-TTCTTGATCCTCGTCCCCAT-3' for nestin, and sense 5'-TGTGTGAAGAGGAAGACAGCAC-3' and antisense 5'-GCACAATACCAGTTGTACGTCC-3' for  $\alpha$ -SMA. After 5 min at 94°C, both reactions were carried out for 35 cycles with cycle times of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, and one cycle of 7 min at 72°C. The amplified products were electrophoresed in 2% agarose gels, stained with ethidium bromide, and photographed. The size of the products obtained was 308 bp for nestin and 412 bp for  $\alpha$ -SMA.

### 2.5. Northern blot analysis

Northern blot assays were performed using 10  $\mu$ g of total RNA per lane and hybridization with  $^{32}$ P-labeled cDNA probes for colla1 and 18S. Autoradiographic signals were quantitated by scanning densitometry.

### 2.6. Western blot analysis of ERK phosphorylation

HSC were treated as described above and extracted in RIPA buffer. Protein concentration of the resultant samples was determined by Bradford assay. For immunoblotting analysis, equal amounts of protein were electrophoresed on sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and transferred on membranes. Membranes were incubated with a specific antibody to ERK (Promega), or actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with the secondary antibody conjugated to horseradish peroxidase (Promega), immunoreactive proteins were detected by enhanced chemiluminescent system (ECL; Amersham International, Little Chalfont, UK).

### 2.7. Western blot analysis of JNK and p38 MAPK phosphorylation

Protein samples were obtained as described above, and samples containing 300  $\mu$ g of total protein were incubated with 1  $\mu$ g anti-phosphotyrosine monoclonal antibody (p-Tyr(PY20); Santa Cruz Biotechnology) for 1 h at 4°C. Immunocomplexes were recovered by adsorption to agarose–protein A (Sigma, St. Louis, MO, USA). After overnight incubation at 4°C, immune complexes were harvested with protein A–agarose, collected by centrifugation and washed four times with RIPA buffer. Bound proteins were released from the particles by boiling for 5 min in SDS–PAGE sample buffer. Proteins were electrophoresed and transferred on membranes and immunoblotted with either anti-JNK or anti-p38 MAPK antibodies (New England Biolabs). Immunoblots were carried out using the ECL method, and signals were quantitated by scanning densitometry.

### 2.8. Statistical analysis

Data were analyzed using the Kruskal–Wallis test to determine differences between all independent groups. When significant differences were obtained ( $P < 0.05$ ), differences between two groups were tested using the Mann–Whitney  $U$ -test.

## 3. Results

### 3.1. TNF- $\alpha$ is not pro-apoptotic for the cell line CFSC-2G

Apoptotic cell death is one of the most common and well characterized effects of TNF- $\alpha$ . However, the ability of TNF- $\alpha$  to induce apoptosis depends on the cell type and in some cases apoptosis only takes place when protein expression has been inhibited at the transcriptional or translational levels [21]. Prior to the study of the antifibrogenic effect of TNF- $\alpha$ , we determined whether TNF- $\alpha$  treatment had an apoptotic effect on the cell line 2G-CFSC. The cytosolic accumulation of oligonucleosomal fragments, that reflects the extent of DNA fragmentation characteristic of apoptosis, was analyzed as a parameter of apoptotic cell death.

Cells were treated with TNF- $\alpha$  (10 ng/ml) for 24 h in the presence or absence of the protein synthesis inhibitor cyclo-

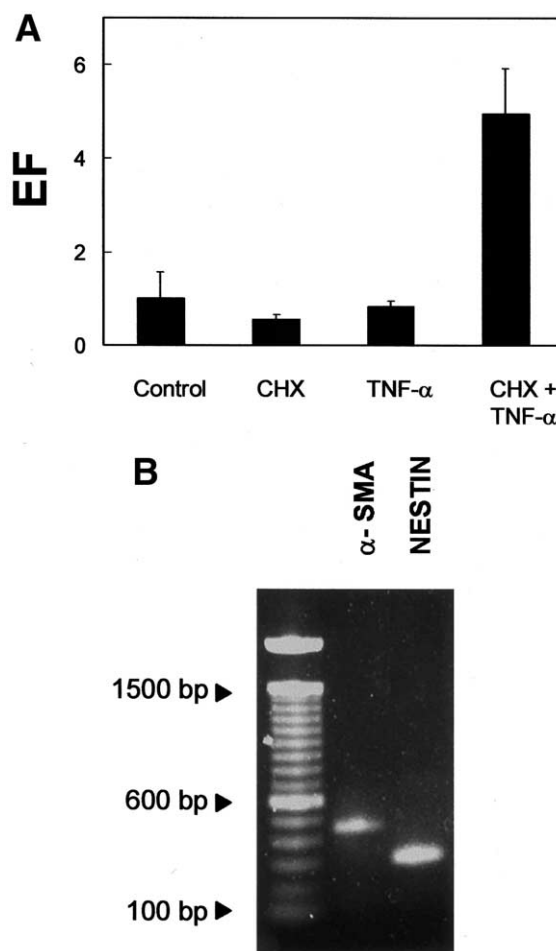


Fig. 1. A: Determination of oligonucleosomal fragments in cytoplasmic extracts from HSC treated with TNF- $\alpha$ , CHX or both. Oligonucleosomal fragments content was expressed as enrichment factor (EF), as described in Section 2. Each bar represents the mean  $\pm$  S.D. of quadruplicate determinations. B: RT-PCR detection of nestin and  $\alpha$ -SMA. Products were resolved on 2% agarose gels and visualized by UV following ethidium bromide staining.

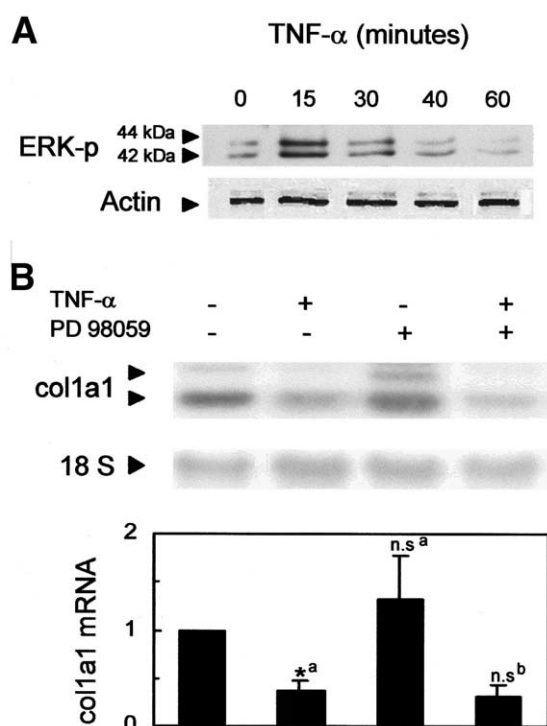


Fig. 2. A: Analysis of ERK phosphorylation in TNF- $\alpha$ -treated HSC. HSC were treated with 10 ng/ml TNF- $\alpha$  for the indicated times and extracts were analyzed by Western blot as described in Section 2. B: Northern blot analysis of collagen mRNA levels of HSC treated with TNF- $\alpha$  and pretreated for 30 min with 10  $\mu$ M PD98059. Each bar represents the mean  $\pm$  S.D. of at least quadruplicate experiments (\* $P < 0.05$ ; a, vs. control; b, vs. TNF- $\alpha$ -treated HSC). Values were corrected for loading differences after hybridization with a cDNA probe for 18S ribosomal RNA. A representative Northern blot with the autoradiographic signals for collagen 1a1 and 18S ribosomal RNA is shown in the upper panel.

heximide (CHX; 100  $\mu$ g/ml). As shown in Fig. 1A, TNF- $\alpha$  had no effect on the accumulation of oligonucleosomal fragments, unless protein synthesis had been blocked by CHX. The resistance of HSC to apoptosis induced by TNF- $\alpha$  has been previously reported and seems to be a consequence of the activation process that takes place as a result of liver damage or when cells are cultured [22]. Activated HSC are characterized by up-regulation of type I collagen and expres-

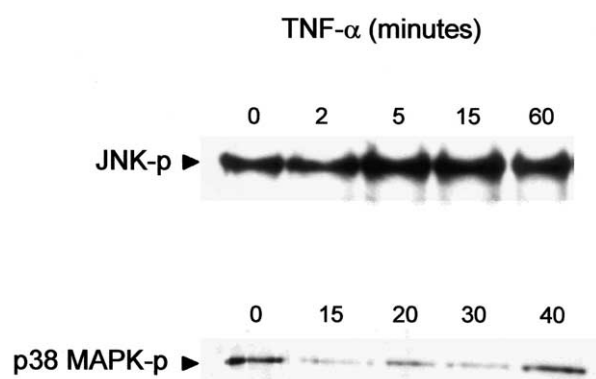


Fig. 3. Analysis of JNK and p38 MAPK phosphorylation in TNF- $\alpha$ -treated HSC. HSC were treated with 10 ng/ml TNF- $\alpha$  for the indicated times and immunoprecipitated extracts were analyzed by Western blot as described in Section 2.

sion of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), nestin and others. The cell line CFSC-2G presents low basal levels of expression of type I collagen genes that can be up-regulated by cytokines and growth factors [15]. However, by RT-PCR we found the presence of messenger RNA for nestin and  $\alpha$ -SMA (Fig. 1B). Therefore, this cell line can be considered as a 'transitional' HSC, in which the activation process is already initiated.

### 3.2. ERK is phosphorylated in response to TNF- $\alpha$

We first studied the role played by the ERK cascade in the inhibitory action of TNF- $\alpha$  on collagen mRNA levels. TNF- $\alpha$  treatment increased ERK1 and 2 phosphorylation 15 min after adding the cytokine (Fig. 2A).

To determine if this effect could be responsible for the down-regulation of collagen elicited by TNF- $\alpha$ , we added the MEK inhibitor PD98059 prior to treatment with TNF- $\alpha$ , and analyzed by Northern blot its effect on collagen mRNA levels.

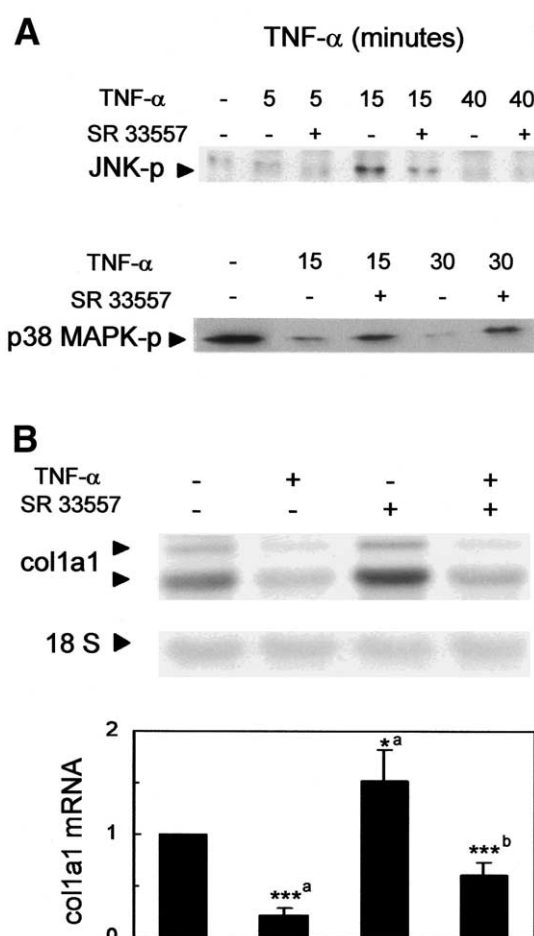


Fig. 4. Effect of the A-SMase inhibitor SR33557. A: Western blot analysis of the effect of SR33557 pretreatment on JNK and p38 MAPK phosphorylation levels in TNF- $\alpha$ -treated HSC. HSC were treated with TNF- $\alpha$  as above and, when indicated, a 30 min pretreatment with 0.1 mM SR33557 was carried out. Results are representative of at least three independent experiments. B: Northern blot analysis of collagen mRNA levels of HSC treated with TNF- $\alpha$  and SR33557. Each bar represents the mean  $\pm$  S.D. of at least quadruplicate experiments (\* $P < 0.05$ , \*\*\* $P < 0.001$ ; a, vs. control; b, vs. TNF- $\alpha$ -treated HSC). Values were corrected for loading differences after hybridization with a cDNA probe for 18S ribosomal RNA. A representative Northern blot with the autoradiographic signals for collagen 1a1 and 18S ribosomal RNA is shown in the upper panel.

As shown in Fig. 2B, PD98059 did not prevent the inhibitory action of TNF- $\alpha$ .

### 3.3. TNF- $\alpha$ decreases p38 MAPK phosphorylation and increases JNK phosphorylation in an A-SMase-dependent fashion

The involvement of enzymes related to the stress-activated cascade, JNK and p38 MAPK, in the effect of TNF- $\alpha$  on HSC was then evaluated. Western blot analysis of time-course treatments with TNF- $\alpha$  revealed a different effect for each enzyme. JNK presented an increased phosphorylation in response to TNF- $\alpha$ , reaching maximum values 5–15 min after treatment, whereas p38 MAPK phosphorylation levels were decreased by TNF- $\alpha$ , being five-fold lower compared to control levels 15 min after treatment (Fig. 3).

To establish if A-SMase mediated either effect, HSC were pretreated with SR33557, a potent A-SMase inhibitor [23]. SR33557 prevented the increased phosphorylation of JNK as well as the decreased phosphorylation of p38 MAPK induced by TNF- $\alpha$  (Fig. 4A), demonstrating both effects to be A-SMase-dependent. Northern blot analysis of the effect of SR33557 on *coll1a1* mRNA levels showed that blockage of A-SMase significantly prevented the inhibitory effect of TNF- $\alpha$  (Fig. 4B).

### 3.4. Inhibition of p38 MAPK down-regulates *coll1a1* mRNA levels

The effect of specific inhibitors for JNK and p38 MAPK was evaluated. HSC were pretreated with SB203580, a pyridinyl imidazole compound that has been described as an inhibitor of p38 MAPK [24], before adding TNF- $\alpha$  to the cell cultures, and *coll1a1* mRNA levels were analyzed by Northern blot. As shown in Fig. 5, SB203580 by itself mimicked the

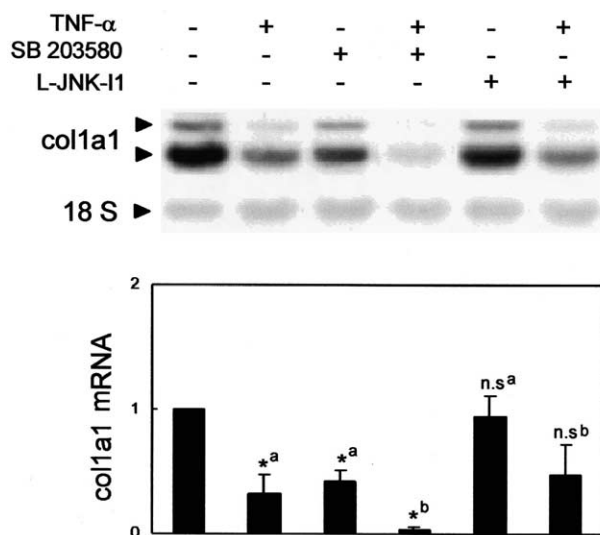


Fig. 5. Effect of p38 MAPK and JNK inhibitors on the down-regulation of *coll1a1* mRNA levels induced by TNF- $\alpha$ . HSC were treated with 10 ng/ml TNF- $\alpha$  for 24 h. When indicated, a 30 min pretreatment with 10  $\mu$ M SB203580 or with 10  $\mu$ M L-JNK-I1 was carried out. Northern blot analysis was performed with 10  $\mu$ g of total RNA. Each bar represents the mean  $\pm$  S.D. of at least quadruplicate experiments (\* $P$  < 0.05; a, vs. control; b, vs. TNF- $\alpha$ -treated HSC). Values were corrected for loading differences after hybridization with a cDNA probe for 18S ribosomal RNA. A representative Northern blot with the autoradiographic signals for *coll1a1* and 18S ribosomal RNA is shown in the upper panel.

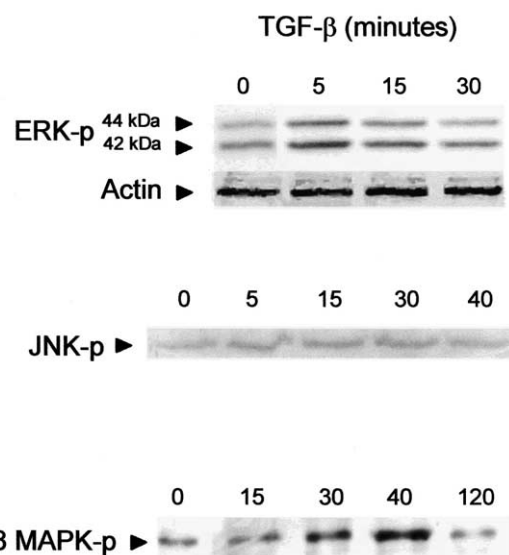


Fig. 6. Time-course study of ERK, JNK and p38 MAPK phosphorylation in TGF- $\beta$ -treated HSC. HSC were treated with 8 ng/ml TGF- $\beta$  for the indicated times and extracts were analyzed by Western blot. Results are representative of at least three independent experiments.

anti-fibrogenic effect of TNF- $\alpha$ , decreasing basal *coll1a1* mRNA levels. An additive inhibitory effect on *coll1a1* mRNA levels was observed in HSC treated with both SB203580 and TNF- $\alpha$ , reaching values of 80% inhibition, as compared to untreated controls. Pretreatment with JNK inhibitor L-JNKI1 had no effect on *coll1a1* mRNA levels, either in control or TNF- $\alpha$ -treated HSC (Fig. 5).

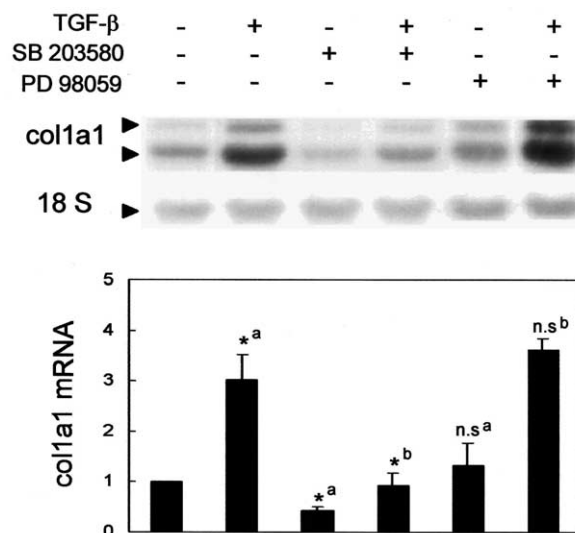


Fig. 7. Effect of p38 MAPK and MEK inhibitors on the up-regulation of *coll1a1* mRNA levels induced by TGF- $\beta$ . HSC were treated with 8 ng/ml TGF- $\beta$  for 24 h. When indicated, a 30 min pretreatment with 10  $\mu$ M SB203580 or 10  $\mu$ M PD98059 was carried out. Northern blot analysis was performed with 10  $\mu$ g of total RNA. Each bar represents the mean  $\pm$  S.D. of at least quadruplicate experiments (\* $P$  < 0.05; a, vs. control; b, vs. TGF- $\beta$ -treated HSC). Values were corrected for loading differences after hybridization with a cDNA probe for 18S ribosomal RNA. A representative Northern blot with the autoradiographic signals for *coll1a1* and 18S ribosomal RNA is shown in the upper panel.



### 3.5. p38 MAPK mediates the TGF- $\beta$ -induced up-regulation of *colla1* mRNA levels

The involvement of p38 MAPK in the regulation of collagen expression by factors different from TNF- $\alpha$  was studied using TGF- $\beta$  as a pro-fibrogenic agent for HSC. Western blot analysis of HSC treated with TGF- $\beta$  showed an increased phosphorylation in both ERK isoforms and in p38 MAPK, while JNK phosphorylation levels remained unchanged (Fig. 6).

The effect of MEK and p38 MAPK inhibitors on *colla1* mRNA was analyzed in TGF- $\beta$ -treated HSC. As previously reported [16], TGF- $\beta$  up-regulated *colla1* mRNA levels.

Pretreatment with SB203580, but not PD98059, prevented the increase of *colla1* mRNA levels induced by TGF- $\beta$  in HSC (Fig. 7). Similar experiments were carried out using the inactive analog SB202474 as a negative control, finding that it had no effect on the action of either TGF- $\beta$  or TNF- $\alpha$  on *colla1* mRNA levels (data not shown).

## 4. Discussion

TNF- $\alpha$  is one of the few cytokines known to produce a decrease in collagen synthesis by activated HSC, and this fact, together with the need for effective anti-fibrogenic therapies, makes the study of the molecular mechanisms responsible for this effect particularly interesting.

The role played by members of the MAPK family on collagen production by HSC has been studied in response to several factors, with different results obtained depending on the agent. ERK cascade has been shown to be related to increased collagen production by HSC in response to IGF-I [25], or as a consequence of the activation process characteristic of this cell type [10]. JNK has been reported to become phosphorylated and activated in HSC in response to UV radiation, leading also to an increased collagen production [14]. Our results indicate that the ERK cascade is activated in HSC by TNF- $\alpha$  and TGF- $\beta$ , but that this activation is not related to the anti- and pro-fibrogenic effects exerted by each cytokine respectively. JNK is also activated by TNF- $\alpha$  in HSC, but this effect does not seem to be responsible for the inhibition of *colla1* mRNA levels, since JNK inhibitor L-JNKI1 did not prevent it.

Several evidences suggest an important role for p38 MAPK in the regulation of collagen production by HSC. Phosphorylation levels of p38 MAPK are higher in activated HSC [26], a phenotype characterized by a dramatic increase in type I collagen synthesis, and inhibition of p38 MAPK both in quiescent and activated HSC has recently been reported to produce a decrease in *colla1* levels [18]. We found that treatment of HSC with TNF- $\alpha$  led to a decreased phosphorylation of p38 MAPK. The correlation between the inhibitory action of TNF- $\alpha$  on *colla1* mRNA and its effect on p38 MAPK phosphorylation levels, and the fact that both effects were A-SMase-dependent, suggested a relationship between the two events. Moreover, inhibition of p38 MAPK by SB203580 mimicked the effect of TNF- $\alpha$  on *colla1* mRNA levels and, added together with TNF- $\alpha$ , produced a higher extent of inhibition. Taken together, these results suggest that activation of A-SMase by TNF- $\alpha$  results in phosphorylation and activation of JNK, and inactivation of p38 MAPK by dephosphorylation, the latter effect leading to *colla1* inhibition.

To establish whether p38 MAPK is also involved in the up-

regulation of collagen production by HSC, we carried out experiments using TGF- $\beta$  as an inducer of collagen expression. TGF- $\beta$  up-regulates collagen expression in several cell types, including cultured HSC [16]. In addition, in other cell types the pro-fibrogenic effect of TGF- $\beta$  has been shown to be mediated by p38 MAPK activation [27,28]. Treatment of HSC with TGF- $\beta$  resulted in an increased phosphorylation of p38 MAPK that correlated with increased *colla1* mRNA levels in response to this cytokine, and the p38 MAPK inhibitor SB203580 prevented the pro-fibrogenic effect of TGF- $\beta$ , strongly suggesting that p38 MAPK participates also in the up-regulation of *colla1* by TGF- $\beta$ .

Recently published data demonstrate that TNF- $\alpha$  and TGF- $\beta$ -responsive elements co-localize in the *colla1* gene promoter [29]. Our findings point to another level of convergence for TNF- $\alpha$  and TGF- $\beta$ , namely, intracellular signal transduction through p38 MAPK phosphorylation. Further studies would be needed to establish whether the transductional and transcriptional effects of these cytokines are related.

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