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CTGF/CCN2 exerts profibrotic action in myoblasts via the up-regulation of sphingosine kinase-1/S1P$_3$ signaling axis: Implications in the action mechanism of TGFβ

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ABSTRACT

The matricellular protein connective tissue growth factor (CTGF/CCN2) is recognized as key player in the onset of fibrosis in various tissues, including skeletal muscle. In many circumstances, CTGF has been shown to be induced by transforming growth factor beta (TGFβ) and accounting, at least in part, for its biological action. In this study it was verified that in cultured myoblasts CTGF/CCN2 causes their transdifferentiation into myofibroblasts by up-regulating the expression of fibrosis marker proteins α-smooth muscle actin and transgelin. Interestingly, it was also found that the profibrotic effect exerted by CTGF/CCN2 was mediated by the sphingosine kinase (SK)-1/S1P$_3$ signaling axis specifically induced by the treatment with the profibrotic cue. Following CTGF/CCN2-induced up-regulation, S1P$_3$ became the S1P receptor subtype expressed at the highest degree, at least at mRNA level, and was thus capable of readdressing the sphingosine 1-phosphate signaling towards fibrosis rather than myogenic differentiation. Another interesting finding is that CTGF/CCN2 silencing prevented the TGFβ-dependent up-regulation of SK1/S1P$_3$ signaling axis and strongly reduced the profibrotic effect exerted by TGFβ, pointing at a crucial role of endogenous CTGF/CCN2 generated following TGFβ challenge in the transmission of at least part of its profibrotic effect. These results provide new insights into the molecular mechanism by which CTGF/CCN2 drives its biological action and strengthen the concept that SK1/S1P$_3$ axis plays a critical role in the onset of fibrotic cell phenotype.

1. Introduction

Inside-out sphingosine 1-phosphate (S1P) signaling represents a common molecular mechanism by which a wide array of extracellular agents, including hormones, cytokines, growth factors and neurotransmitters elicit, at least in part, their biological effects [1–3]. The molecular machinery necessary to activate in autocrine and/or in paracrine fashion one or more S1P receptor subtypes comprises sphingosine kinase(SK)-1, or less frequently SK2, both responsible for the intracellular formation of the bioactive sphingolipid S1P, and a membrane transporter that facilitates the release of generated S1P in the extracellular compartment [4]. Then activated S1P receptors, which are coupled to multiple heterotrimeric GTP binding proteins, coordinate the activation of numerous signaling pathways, thus accounting for the final biological response [5]. A wealth of experimental data support a key role of inside-out S1P signaling downstream of primary extracellular cues in the control of cell migration, proliferation and differentiation in a variety of cellular settings. In this regard, it has been firmly established that transforming growth factor beta (TGFβ) exploits S1P signaling axis to exert its profibrotic effect in fibroblasts. Indeed, SK1 is up-regulated by TGFβ in dermal fibroblasts and its expression is required for the induction of the tissue inhibitor of metalloproteases-1 that favors the deposition of extracellular matrix proteins by reducing their degradation [6]. Accordingly, it has been proved that SK1 and S1P$_2$ are implicated in TGFβ-dependent biosynthesis of collagen in cardiac fibroblasts [7]. Moreover, TGFβ-induced transdifferentiation of lung fibroblasts into myofibroblasts, which plays a major role in the onset of pulmonary fibrosis, was found to rely on SK1 and both S1P$_2$ and S1P$_3$ [8].

Besides its major role in the transmission of the fibrotic effect of TGFβ in fibroblasts, S1P signaling has been also individuated as critical

Abbreviations: CTGF, connective tissue growth factor; TGFβ, transforming growth factor beta; SK, sphingosine kinase; S1P, sphingosine 1-phosphate; α-SMA, α-smooth muscle actin; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's modified phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumin; siRNA, short interfering RNA; ECL, enhanced chemiluminescence; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SCR, scrambled; PI, propidium iodide

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player in the onset of fibrotic phenotype in skeletal muscle precursor cells such as myoblasts. TGFβ was found to induce transdifferentiation of myoblasts into myofibroblasts, in turn responsible for extracellular matrix protein deposition, progressive replacement of the muscle fibers with connective tissue and impairment of skeletal muscle repair [9]. Notably, in cultured myoblasts S1P endogenously generated by SK1, via the ligation of $S1P_2$, participates to the correct accomplishment of myogenic program that drives their differentiation into myotubes, multinucleated cells endowed with contractile properties that resemble skeletal myofibres [10]. In accordance, insulin-like growth factor-1, pivotal physiological regulator of myogenesis, exerts at least in part its biological action via SK1 activation and $S1P_2$ engagement [11]. In contrast, TGFβ is capable of fully readdressing the inside-out signaling of S1P in myoblasts, by up-regulating SK1, that accounts for increased S1P production and enhancing the expression of $S1P_3$ that, becoming the highest expressed receptor subtype, acquires a dominant signaling action [9].

Although the SK1/$S1P_3$ axis has been identified as key player in the profibrotic action of TGFβ in myoblasts, at present is unknown whether it is implicated in the mechanism by which other profibrotic cues exert their biological action. Here, to address the overall relevance of the SK1/$S1P_3$ axis, we have examined whether this signaling pathway is downstream to another key inducer of skeletal muscle fibrosis such as connective tissue growth factor (CTGF), also known as CCN2, a member of the CCN family of secreted matricellular proteins. This protein, produced

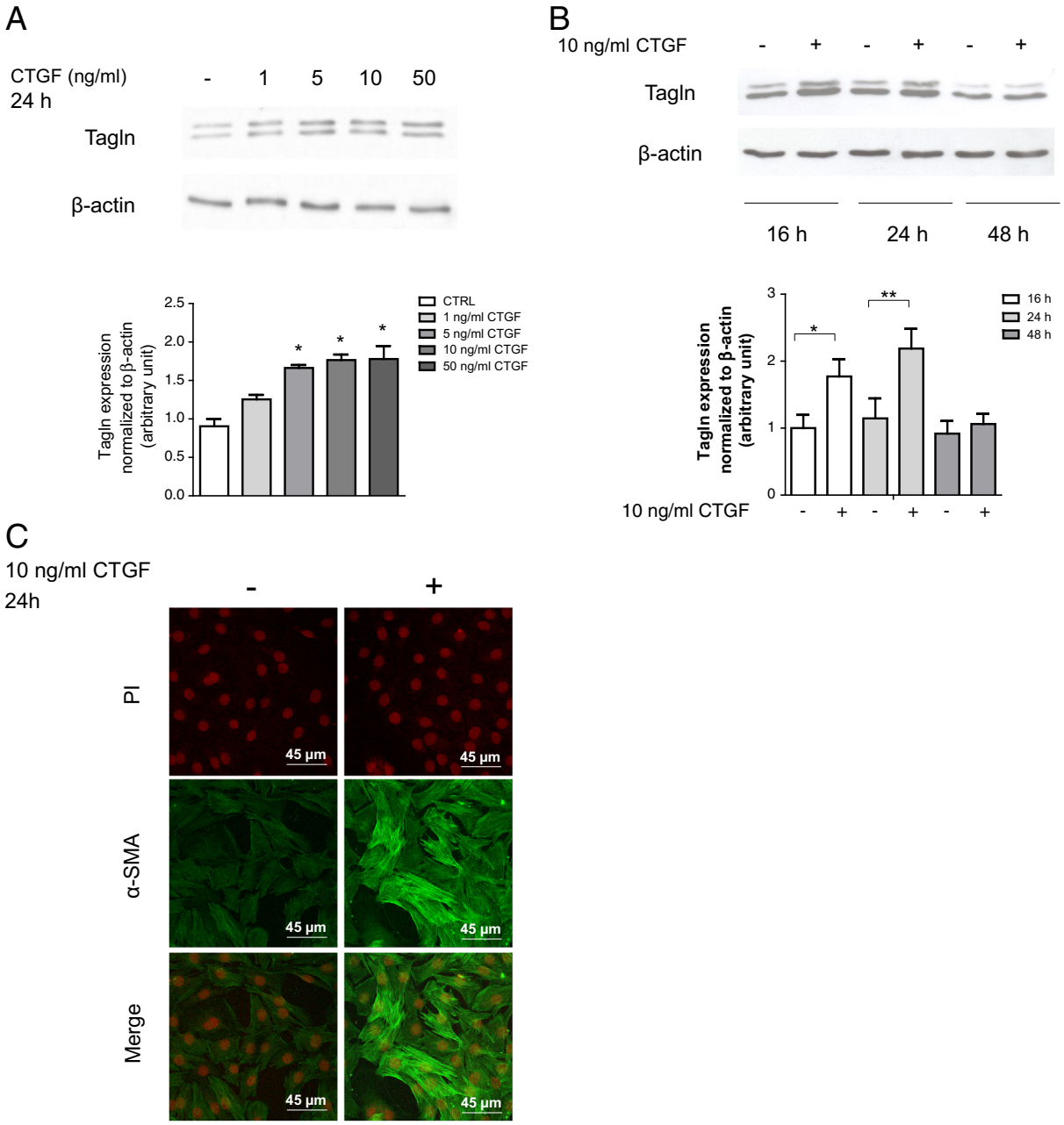

Fig. 1. CTGF exerts a profibrotic action in C2C12 myoblasts. (A) Dose–response effect of CTGF on the transdifferentiation of myoblasts into myofibrobalsts is evaluated after treatment with the indicated concentration of CTGF by analyzing the expression of the fibrosis marker Transgelin (Tagln) in 10 μg of protein in total cell lysate. Upper panel, Western blot analysis is performed by using specific anti-Tagln antibody. Lower panel, results are obtained by densitometric analysis, normalized to β-actin content in each specimen, and data are reported as mean ± SEM of three independent experiments, fold change compared to control, set as one. The effect of CTGF is statistically significant by one-way ANOVA, followed by Bonferroni's post hoc test, $^{*}P < 0.05$. (B) Upper panel, time-dependent effect of CTGF in myoblasts is analyzed by measuring the expression of Tagln at the indicated time points. Lower panel, results are analyzed and reported as described in section A. The effect of CTGF at 16 h and 24 h is statistically significant by one-way ANOVA, followed by Bonferroni's post hoc test, $^{*}P < 0.05$, $^{**}P < 0.01$. (C) Immunofluorescence analysis of the fibrosis marker α-smooth muscle actin (α-SMA) is performed in myoblasts treated with CTGF for 24 h, using a specific mouse anti-α-SMA antibody, fluorescein-conjugated anti-mouse secondary antibody and propidium iodide (PI) staining (images are representative of three separate experiments).