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Inhibitory effects of PPAR γ ligands on TGF- β 1-induced CTGF expression in cat corneal fibroblasts

Kye-Im Jeon¹, Richard P. Phipps^{1,2,3}, Patricia J. Sime^{2,3}, and Krystel R. Huxlin^{1,4}

¹Flaum Eye Institute, University of Rochester, Rochester NY, USA

²Department of Environmental Medicine, University of Rochester, Rochester NY, USA

³Department of Medicine, University of Rochester, Rochester NY, USA

⁴Center for Visual Science, University of Rochester, Rochester NY, USA

Abstract

Ligands of Peroxisome Proliferator Activated Receptor gamma (PPAR γ) possess strong anti-fibrotic properties in the cornea and several other body tissues. In the cornea, we recently showed this class of molecules to prevent stromal myofibroblast differentiation partially by blocking the actions of p38 mitogen-activated protein kinase (MAPK). However, given the important role assigned to connective tissue growth factor (CTGF) in mediating corneal fibrosis, here we asked whether PPAR γ ligands also act by affecting transforming growth factor- β (TGF- β 1-induced expression of CTGF in cultured corneal fibroblasts. Corneal keratocytes were isolated from young, adult cats and early passage cells were exposed to TGF- β 1 with or without the PPAR γ ligands Rosiglitazone, Troglitazone and 15d-PGJ2. Western blots were used to assay levels of CTGF and alpha smooth muscle actin (α SMA), a marker of myofibroblast differentiation. CTGF siRNA demonstrated a critical role for CTGF in TGF- β 1-mediated myofibroblast differentiation, while exogenously applied CTGF potentiated the pro-fibrogenic effects of TGF- β 1. TGF- β 1-mediated increases in CTGF and α SMA expression were strongly inhibited by all three PPAR γ ligands tested, and by a c-jun N-terminal kinase (JNK) inhibitor. However, while extracellular signal-regulated kinase (ERK) 1/2, protein kinase B (AKT) and p38 MAPK inhibitors also blocked TGF- β 1-induced α SMA induction, they did not dampen TGF- β 1-induced increases in levels of CTGF. Thus, we conclude that PPAR γ ligands block TGF- β -induced increases in CTGF levels in cat corneal fibroblasts. They appear to do this in addition to their anti-fibrotic effect on p38 MAPK, providing a second intracellular pathway by which PPAR γ ligands block α SMA induction.

Correspondence: Dr. Krystel R. Huxlin, PhD, Flaum Eye Institute, Box 314; University of Rochester Medical Center; 210 Crittenden Blvd, Rochester, NY 14642, USA; phone +1-(585) 275- 5495; huxlin@cvs.rochester.edu.

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Keywords

Rosiglitazone; Troglitazone; 15d-PGJ2; JNK; p38 MAPK; ERK; AKT

1. Introduction

Following damage to the corneal surface, surviving keratocytes are exposed to multiple wound healing modulators - including transforming growth factor- β (TGF- β - the strongest known pro-fibrotic agent (Jester et al 1996, Vesaluoma et al 1997). As a result, keratocytes adjacent to the damaged zone proliferate, migrate, transform into fibroblasts (Fini 1999) and then, into myofibroblasts (Jester et al 1987). Activated TGF- β receptors appear to regulate multiple genes that control the differentiation of fibroblasts into myofibroblasts, and the actions of myofibroblasts within their immediate extracellular matrix (ECM) environment (Desmouliere et al 1993, Jester et al 1995, Wilson 2012). One molecule thought to regulate many of the cell-ECM interactions during fibrosis and scar formation is connective tissue growth factor (CTGF) (Garrett et al 2004). TGF- β has been shown to directly increase CTGF mRNA and protein levels within cells (Blalock et al 2003, Igarashi et al 1996, Kikuchi et al 1995, Tall et al 2010).

CTGF is a major autocrine growth factor, which mediates the actions of TGF- β with respect to myofibroblast differentiation and behavior (Grotendorst 1997, Grotendorst et al 1996, Lipson et al 2012). CTGF is a secreted, cysteine-rich molecule approximately 36–38kD in size, which was initially identified in media conditioned by human umbilical vein endothelial cells, as a new fibroblast mitogen (Bradham et al 1991). CTGF is also known as CCN2, as it belongs to the CCN (cyr61, ctgf, neuroblastoma over-expressed gene (nov)) family, an important set of matrix-cellular regulatory factors involved in internal and external cell signaling (Moussad & Brigstock 2000). This family participates in angiogenesis, chondrogenesis, and wound healing (Perbal 2004), and appears to be a central mediator of tissue remodeling and fibrosis. TGF- β directly regulates the expression of CTGF in primary skin fibroblasts (Igarashi et al 1993), human pulmonary fibroblasts (Wang & Liu 2006), and in corneal fibroblasts (Folger et al 2001). Histological evaluation of corneal scar formation in pseudophakic bullous keratopathy showed an increase in CTGF expression and in the number of TGF- β -positive stromal cells (Liu et al 2012). Furthermore, CTGF protein and mRNA levels increased in rat cornea through day 21 after photorefractive keratectomy (PRK) (Blalock et al 2003). Therefore, CTGF appears to be an important effector molecule in both physiological and pathological processes associated with fibrosis (Wahab et al 2001) and may provide a new target for therapeutic interventions to mitigate fibrotic diseases (Moussad & Brigstock 2000).

In the cornea, fibrosis and scarring lead to vision loss, and remain without effective treatment. A solution would be a drug that selectively targets the TGF- β cascade, but with minimal side-effects. Our group and others have shown several ligands of PPAR γ to be effective corneal anti-fibrotics *in vitro* (Jeon et al 2014, Kuriyan et al 2012, Pan et al 2009, Pan et al 2011) and *in vivo* (Huxlin et al 2013, Jeon et al 2014). PPAR γ is a transcription factor belonging to a nuclear receptor superfamily that regulates important cellular functions, including metabolism, adipogenesis, proliferation, differentiation and

inflammatory responses (Simpson-Haidaris et al 2010). A significant body of data suggests that PPAR γ ligands can act as anti-fibrotics in a range of body tissues, including lung (Ferguson et al 2009, Lin et al 2010, Zhou et al 2012), skin (Ghosh et al 2004), kidneys (Liu et al 2011) and cornea (Huxlin et al 2013, Jeon et al 2014, Kuriyan et al 2012, Pan et al 2009, Pan et al 2011). In the cornea, two such ligands - Rosiglitazone and Troglitazone – were recently shown effective at control fibrosis after PRK, without significant side-effects (Huxlin et al 2013, Jeon et al 2014). Here, we asked whether PPAR γ ligands exert part of their anti-fibrotic actions in the cornea by influencing TGF- β 1-induced CTGF expression in stromal fibroblasts - a potentially novel mechanism of action for this promising group of molecules.

2. Materials and methods

All animal procedures were conducted according to the guidelines of the University of Rochester Committee on Animal Research (UCAR), the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the NIH Guide for the Care and Use of Laboratory Animals.

2.1. Isolation and culture of cat corneal fibroblasts

Primary feline corneal keratocytes were isolated as previously described (Huxlin et al 2013, Jeon et al 2014). In brief, fresh corneas were obtained immediately post-mortem from young, adult domestic short-hair cats (*felis catus*). The corneal epithelium and endothelium were scraped off. After double enzyme digestion, the stroma was centrifuged at 500 rpm for 2 min at 4°C and the pellet removed. The resulting supernatant, containing mostly keratocytes, was centrifuged again at 3000 rpm for 10 min and was re-suspended in 2 ml of Fibroblast Growth Factor-containing medium (PromoCell GmbH, Germany), counted and seeded onto culture plates (Greiner Bio-one; Monroe, NC). All experiments were performed in triplicate.

2.2. Measuring the effect of TGF- β 1 on CTGF and α SMA expression

Passage 6–7 feline, corneal fibroblasts were seeded at a density of 1×10^5 – 2×10^5 cells/ 6-cm dish containing DMEM/F12 + 5% HS. The medium was changed to one containing DMEM/F12 + 1% HS for 1 day in order to promote cellular quiescence. Western blots were used to detect the expression of CTGF and α SMA relative to the expression of β -Tubulin as a loading control, as described previously (Huxlin et al 2013, Jeon et al 2014). Expression of α SMA was used as positive control to indicate myofibroblast differentiation. A 10% gel was run using 10–20 μ g of cell lysates separated by electrophoresis, transferred to a nitrocellulose membrane and probed with primary antibodies - rabbit polyclonal anti-CTGF (1:1000, from ab6992 Abcam Inc. Cambridge, MA); mouse monoclonal anti- α SMA, (1:10,000, from Thermo Fisher Scientific, Pittsburgh, PA) and mouse monoclonal β -Tubulin (1:5000, from Santa Cruz Inc., Santa Cruz, CA). Primary antibodies were incubated overnight at 4°C, the membranes rinsed and secondary antibodies (anti-mouse IgG or anti-rabbit IgG-horse radish peroxidase, GE Healthcare; Piscataway, NJ) were applied and incubated for 1hr at room temperature. The membranes were scanned with a Chemi-doc

machine (Bio-Rad, Hercules, CA) and the resulting images were imported into Image J (NIH) for measurement of relative protein expression.

2.3. Effect of CTGF siRNA on TGF- β 1-induced CTGF and α SMA expression

To evaluate the role of CTGF in TGF- β 1-induced myofibroblast differentiation, we used CTGF siRNA (5'-GAGAGACATTAACCTCATT-3', cat. J-012633-13-05, GE Dharmacon) to knock-down expression of CTGF. Non-targeting siRNA (cat. D-001810-01-05, GE Dharmacon) was used as a negative control. Lipofectamine RNAiMAX (Invitrogen) reagent was used to perform siRNA transfections as per the manufacturer's instructions. Briefly, 1×10^5 cells/6-well plate were seeded in DMEM/F12 with 5% HS. After attachment, cells were changed into reduced serum-containing media for 1 day, then transfected with CTGF siRNA or control siRNA. About 24 hours after transfection, cells were treated with recombinant human TGF- β 1 (1 ng/ml, R&D Systems, Inc.; Minneapolis, MN). Two days later, cells were harvested and Western blots were used to estimate the expression of CTGF and α SMA relative to that of β -Tubulin.

2.4. Effect of exogenous CTGF on time-dependent α SMA expression

To assess whether exogenously-provided CTGF could induce the expression of pro-fibrotic molecules in corneal fibroblasts independent of TGF- β 1, corneal fibroblasts were seeded at a density of 5×10^4 cells/well in 6-well plates containing DMEM/F12 + 5% HS. After attachment, the medium was changed to one containing DMEM/F12 + 1% HS for 1 day in order to promote cellular quiescence. Cells were treated with 50ng of recombinant, C-terminus CTGF (Shenandoah Biotechnology, Warwick, PA) with or without 1ng/ml recombinant human TGF- β 1 for indicated times. Western blots were used to quantify the expression of α SMA relative to that of β -Tubulin.

2.5. Effect of PPAR γ ligands on TGF- β 1-induced CTGF expression

We tested the effects of 3 different PPAR γ ligands - Troglitazone, Rosiglitazone and 15-deoxy-delta12, 14-prostaglandin J2 (15d-PGJ2) - on CTGF expression after TGF- β 1 stimulation. Cells were pre-treated with optimal doses of the three PPAR γ ligands of interest, determined to decrease TGF- β 1-induced expression of α SMA, COL1 and FN in cultured feline corneal fibroblasts in a prior *in vitro* study using identical cell culture conditions (Jeon et al 2014). Either 15 μ M Troglitazone (Cayman; Ann Arbor MI), 75 μ M Rosiglitazone (Cayman; Ann Arbor MI), or 5 μ M 15d-PGJ2 (Enzo; Plymouth Meeting, PA) were applied to the cells in 1% HS in DMEM/F12 medium for 30 min. TGF- β 1 (1 ng/ml) was added to the culture medium. Cells were harvested 1 day later and Western blots were used to quantify expression of CTGF relative to that of β -Tubulin, as described earlier.

2.6. Effect of kinase inhibitors on TGF- β -induced CTGF and α SMA expression

In order to assess whether the molecular pathways mediating the effects of PPAR γ ligands on CTGF were similar to those impacting myofibroblast differentiation, we used small-molecule kinase inhibitors to block four major, known, endogenous, pro-fibrotic protein kinases (Mu et al 2012). Specifically, we used the ERK inhibitor U0126 (Marampon et al 2011), the p38 MAPK inhibitor SB203580 (Barancik et al 2001), the AKT inhibitor

LY294002 (Gharbi et al 2007) and the JNK inhibitor SP600125 (Wang et al 2007). All inhibitors were obtained from Calbiochem (San Diego, CA). Passage 6–7 cells were pre-treated for 30 mins with optimal doses of each kinase inhibitor (determined previously (Jeon et al 2014)), except for the JNK inhibitor, for which different doses had to be tested anew. All inhibitors were dissolved in 1% HS-DMEM/F12. After 30 mins, 1 ng/ml of TGF- β 1 was added to the medium and the cells were incubated for 3 days, at which point Western blots were performed to assess the expression of CTGF relative to that of β -Tubulin. Expression of α SMA was used as a positive control for myofibroblast differentiation

2.7. Statistical analysis

In order to estimate differences in protein expression levels on Western blots, when three or more groups were compared, inter-group differences were tested with an ANOVA. When only two groups were compared, a two-sided Student's t-test was performed. A probability of error of $p < 0.05$ was considered statistically significant. All statistical tests were performed using the SPSS 20.0.0 software package (SPSS Inc., Chicago, Ill.).

3. Results

3.1. TGF- β 1 increases expression of CTGF before α SMA

In the absence of TGF- β stimulation, basal levels of CTGF expression in cultured corneal fibroblasts were low but distinctly above zero (Fig. 1A, first lane), suggesting that CTGF was constitutively synthesized and expressed at low levels in culture. Addition of TGF- β 1 to the culture medium induced a gradual increase in levels of CTGF protein relative to the untreated condition, starting within 1 hour and peaking at 2–3 days after onset of TGF- β 1 treatment (Fig. 1A, B). Of note is the faster rate of increase of CTGF expression relative to that of α SMA over the first 24 hrs after TGF- β 1 stimulation. During this time, α SMA levels remained almost flat, exhibiting the first significant increase in expression on the second day after TGF- β 1 stimulation. A repeated measured ANOVA confirmed this, with a significant main effect of time in culture [F (8,32)=42.519, $p < 0.0001$], a significant main effect of CTGF/ α SMA expression [F (1,4)=9.752, $p = 0.035$] and a significant interaction between the two [F (8,32)=5.426, $p < 0.0001$].

3.2. CTGF siRNA blocks TGF- β 1-induced CTGF and α SMA expression

After 2 days in culture, corneal fibroblasts transfected with control siRNA, showed strong TGF- β 1-induced increases in the expression of CTGF and α SMA relative to un-stimulated fibroblasts (Fig. 2A, B). In contrast, CTGF siRNA suppressed TGF- β 1-induced CTGF expression by about $47 \pm 16\%$ (mean \pm SEM) relative to the control siRNA + TGF- β 1 condition. This blockage of CTGF expression was associated with a $52 \pm 15\%$ decrease in α SMA expression relative to control siRNA cells stimulated with TGF- β 1 alone (Fig. 2A, B). In contrast, total β -tubulin levels were unaffected by addition of either TGF- β 1- or siRNA (Fig. 2A). These data suggest that CTGF contributes significantly to TGF- β 1-induced cat corneal myofibroblast differentiation.

3.3. CTGF potentiates the pro-fibrotic effects of TGF- β 1

Next, we assessed whether exogenously supplied CTGF was able to induce corneal myofibroblast differentiation in the absence of TGF- β 1 stimulation. As shown in Fig. 2C, CTGF alone did not increase expression of α SMA when used from 1–3 days in culture. Even the highest dose tested –200ng/ml - was unsuccessful at inducing myofibroblast differentiation (data not shown). However, combined exposure of corneal fibroblasts to 1 ng/ml TGF- β 1 together with 50ng/ml of CTGF potentiated α SMA expression above levels seen following incubation with 1 ng/ml TGF- β 1 alone. A repeated-measures ANOVA contrasting CTGF+TGF- β 1 with TGF- β 1 treatment across the 3 days of culture revealed a significant effect of time [F(2,17)=46.5, p <0.0001] and treatment [F(1, 17)=10, p =0.0341] but no significant interaction between time and treatment.

3.4. PPAR γ ligands inhibit CTGF expression

All three PPAR γ ligands tested (two synthetic ligands - Rosiglitazone, Troglitazone, and the natural ligand 15d-PGJ2) inhibited TGF- β 1-induced increases in the expression of CTGF after 1 day in culture (Fig. 3A–C). In all cases, levels of CTGF returned back to baseline (i.e., pre-TGF- β 1) levels. Importantly, these effects occurred at doses of the three PPAR γ ligands, which were effective at blocking up-regulation of α SMA expression (Jeon et al 2014). In contrast, total β -tubulin levels were unaffected by addition of either TGF- β 1 or PPAR γ ligands (Fig. 3A–C).

3.5. PPAR γ ligand effects on CTGF are not mediated by p38 MAPK

Prior work in our laboratory showed PPAR γ ligands to exert their anti-fibrotic effects in corneal fibroblasts by blocking phosphorylation of p38 via the p38 MAPK (Jeon et al 2014). To ascertain whether this or other signaling mechanism(s) were involved in regulation of CTGF levels, we used small-molecule inhibitors to block several key endogenous protein kinases, known to be responsible for phosphorylation of p38, Akt, ERK and JNK. We contrasted the impact of these kinase inhibitors on CTGF expression with the impact of one of the PPAR γ ligands (75 μ M Rosiglitazone) previously shown to inhibit corneal fibrosis by inhibiting phosphorylation of p38 (Jeon et al 2014). As shown in Fig. 4A, basal CTGF levels were low but detectable in passage 6–7 corneal fibroblasts (Lane 1). Addition of 1 ng/ml TGF- β 1 for 3 days increased both CTGF and α SMA expression (Lane 2, Fig. 3B). Pre-incubation with 75 μ M Rosiglitazone decreased the TGF- β -induced increases in CTGF and α SMA expression (Lane 3, Fig. 4A). While administration of p38, ERK and AKT kinase inhibitors decreased α SMA expression (as reported in our prior work (Jeon et al 2014)), none of them blocked the TGF- β 1-induced increase in CTGF expression (Lanes 4–6, Fig. 4A). As a positive control, we tested an inhibitor of JNK. JNK signaling was previously shown to be critical for CTGF expression and scar formation in the cornea (Chang & Wu 2009, Chang & Wu 2010, Radhakrishnan et al 2012, Shi et al 2012). Consistent with this prior work, administration of SP600125, which inhibits phosphorylation of JNK, blocked TGF- β 1-induced increases in the expression of both CTGF and α SMA in a dose-dependent manner (Figs. 4B, 5).

4. Discussion

Corneal injury can lead to scar formation by way of corneal fibrosis, which involves the appearance of myofibroblasts and the improper deposition of ECM components (Wilson 2012). TGF- β is perhaps the most important, pro-fibrotic growth factor. It is known to stimulate myofibroblast differentiation and its associated phenotype, which includes the production of α SMA and ECM molecules. However, gaps in knowledge remain with respect to the molecular mechanisms of fibrosis in different body tissues, and how best to control them. Corneal scarring is a predominant affliction worldwide, for which there is no effective treatment without side-effects, and whose consequence when untreated and uncontrolled, can be blindness (Whitcher et al 2001). Finding a safe way of controlling fibrosis in the cornea would be of tremendous public health benefit.

CTGF has been recognized as a principal downstream mediator of TGF- β -induced fibroblast proliferation and differentiation into myofibroblasts (Grotendorst 1997, Grotendorst et al 1996, Lipson et al 2012). As summarized in Fig. 5, in corneal fibroblasts, TGF- β stimulation increases CTGF mRNA and protein levels (Tall et al 2010), and with respect to protein expression, we replicated this finding here. However, we also noted that TGF- β -induced increases in CTGF expression preceded increases in α SMA expression, supporting the notion that CTGF is produced in response to TGF- β as an immediate early gene product (Folger et al 2001, Grotendorst et al 1996). In addition, we confirmed that TGF- β regulates CTGF levels in cat corneal fibroblasts via JNK signaling (Fig. 5), consistent with prior studies in telomerase-immortalized human corneal stromal fibroblasts (Chang & Wu 2009, Chang & Wu 2010, Shi et al 2012) and in quiescent human corneal fibroblasts (Radhakrishnan et al 2012).

CTGF is a modular protein, containing up to four distinct functional domains that can bind diverse growth factors and cytokines (Leask & Abraham 2006). Therefore, this single molecule can modify a range of signal transduction pathways, depending on the particular partners or combination of partners with which it interacts. For example, CTGF binds TGF- β through its N-terminus domain, thus playing an important role in augmenting TGF- β activity (Leask & Abraham 2006). However, there is less information regarding CTGF's other physiological interactions and effects, although epidermal growth factor (EGF) and insulin-like growth factor (IGF)-2 are both known to influence CTGF-mediated cell proliferation and differentiation (Grotendorst & Duncan 2005).

Curiously, while CTGF appears to control many cell-ECM interactions during fibrosis and scar formation, prior work showed that CTGF alone was not sufficient to induce myofibroblast differentiation, α SMA expression or collagen matrix contraction in human corneal fibroblast (Garrett et al 2004). We confirmed that CTGF alone was not sufficient to induce a pro-fibrotic response in cat corneal fibroblasts, but exogenously-supplied CTGF potentiated the increased expression of α SMA resulting from TGF- β 1 stimulation. As a result, we decided to use siRNA to critically evaluate the role of CTGF in TGF- β 1-induced myofibroblast differentiation of cat corneal fibroblasts. We found that CTGF knockdown blocked TGF- β 1-induced increases in α SMA expression. Of relevance here, CTGF-deficient mouse embryonic fibroblasts exhibit a significant reduction in focal adhesion kinase,

PI3kinase, Akt and ERK phosphorylation, in addition to delays in cell spreading and formation of actin stress fibers (Chen et al 2004). Moreover, loss of CTGF expression also impairs the ability of TGF- β to induce type 1 collagen and α SMA expression in such cells (Shi-wen et al 2006). All in all, these results support the notion that CTGF may be a key co-factor, required for TGF- β to induce the signals needed for myofibroblast differentiation. In the absence of CTGF, corneal fibroblasts appear to become refractory to pro-fibrotic stimuli like TGF- β .

One interesting point of note here is that we used two different methods to block the expression of CTGF (a JNK inhibitor and siRNA system) and found them to have a slightly different magnitude/rate of impact on α SMA expression. CTGF siRNA decreased α SMA expression by almost half (Fig. 2A), whereas the JNK inhibitor appeared to completely prevent TGF- β 1-induced α SMA expression at very low doses (Fig. 4B). One interpretation of these findings is that the siRNA knockdown was less effective (and transient) as a blocker, and took some time to exert its effect after induction. In contrast, small molecule inhibitors with proven selectivity (such as the one used for JNK) can easily access all cells in a culture, and because they are membrane permeable, can diffuse very rapidly inside cells, providing inhibition fast, selectively and even irreversibly (if covalently bound to their targets).

Having established that CTGF plays an important role in corneal myofibroblast differentiation, our next question was: do PPAR γ ligands exert some of their anti-fibrotic actions in the cornea because they prevent TGF- β 1-induced increases in CTGF levels inside corneal fibroblasts? Recent work from our laboratory showed Rosiglitazone and Troglitazone – two synthetic PPAR γ ligands - to effectively control fibrosis post-PRK without significant side-effects; as summarized in Fig. 5, *in vitro*, these ligands were shown to act at least partially, by inhibiting p38 MAPK (Huxlin et al 2013, Jeon et al 2014). Here, we report that PPAR γ ligands also block TGF- β 1-induced increases in CTGF levels in cultured corneal fibroblasts - an effect that appears to be independent of the ligands' actions on p38 MAPK. Indeed, inhibiting this kinase did not change levels of CTGF in stimulated, cultured fibroblasts. One possibility is that PPAR γ ligands block CTGF up-regulation by inhibiting JNK, as in adipocytes (Diaz-Delfin et al 2007). Another possibility (not mutually exclusive) is that PPAR γ ligands decrease synthesis of CTGF receptors, thus reducing the uptake of this growth factor from the extracellular milieu. There are several possible candidates for this role, including the low-density lipoprotein receptor-related protein-1 (LRP1) (Kawata et al 2006, Wahab et al 2001) and the mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF-2-R) (Blalock et al 2012). Blalock and colleagues (2012) reported that M6P/IGF-2-R was the most likely receptor to regulate CTGF-stimulated *proliferation* of human corneal fibroblasts. In contrast, CTGF binding of LRP1 is the major event regulating ECM remodeling and fibrosis in cartilage (Kawata et al 2010). In fact, LRP1 mediates the uptake and intracellular transport of CTGF in chondrocytes (Wahab et al 2001) and in human pulmonary fibroblasts, TGF- β 1 stimulation increases the expression of both CTGF and LRP1 (Wang & Liu 2006). However, the nature of the CTGF receptor(s) in corneal fibroblasts remains to be determined, and as such, we can only speculate as to whether PPAR γ ligands are likely to regulate it.

In conclusion, the present results show that in cultured, feline, corneal fibroblasts, the expression of CTGF is rapidly increased by TGF- β 1 stimulation. At least in part, this occurs via regulation of JNK signaling (Fig 5). PPAR γ ligands blocked the up-regulation of CTGF at doses that prevented myofibroblast differentiation and increased expression of pro-fibrotic molecules such as α SMA, Fibronectin and Collagen 1. Details about the interactions between CTGF and other pro-fibrotic signaling pathways (Akt, ERK, p38 MAPK) remain to be elucidated, as do regulatory mechanism by which PPAR γ ligands block TGF- β 1-induced up-regulation of CTGF. However, because CTGF appears to be a critical mediator of TGF- β 's pro-fibrotic effects in the cornea, our findings present a novel mechanism of action by which PPAR γ ligands exert their anti-fibrotic actions in an organ whose transparency is essential for our sense of sight.

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Abbreviations

CTGF	connective tissue growth factor
CCN	cyr61, ctgf, neuroblastoma over-expressed gene
Nov	neuroblastoma over-expressed gene
ECM	extracellular matrix
PRK	photorefractive keratectomy
PPARγ	peroxisome proliferator activated receptor gamma
15d- PGJ2	15-deoxy-delta 12,14-prostaglandin J2
UCAR	University of Rochester Committee on Animal Research
TGF-β	transforming growth factor-beta
αSMA	alpha smooth muscle actin
MAPK	mitogen-activated protein kinase
JNK	c-jun N-terminal kinase
AKT	protein kinase B
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
LPR1	low-density lipoprotein receptor-related protein-1
M6P/IGF-2-R	mannose 6-phosphate/insulin-like growth factor2 receptor
EGF	epidermal growth factor

(IGF)-2	insulin-like growth factor
MEFs	mouse embryonic fibroblasts
FAK	focal adhesion kinase

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Highlights

- TGF- β 1 increases the expression of CTGF and α SMA in cultured cat corneal fibroblasts
- CTGF siRNA blocks TGF- β 1-induced myofibroblast differentiation in corneal fibroblasts
- PPAR γ ligands block TGF- β 1-induced up-regulation of CTGF, independently of p38 MAPK

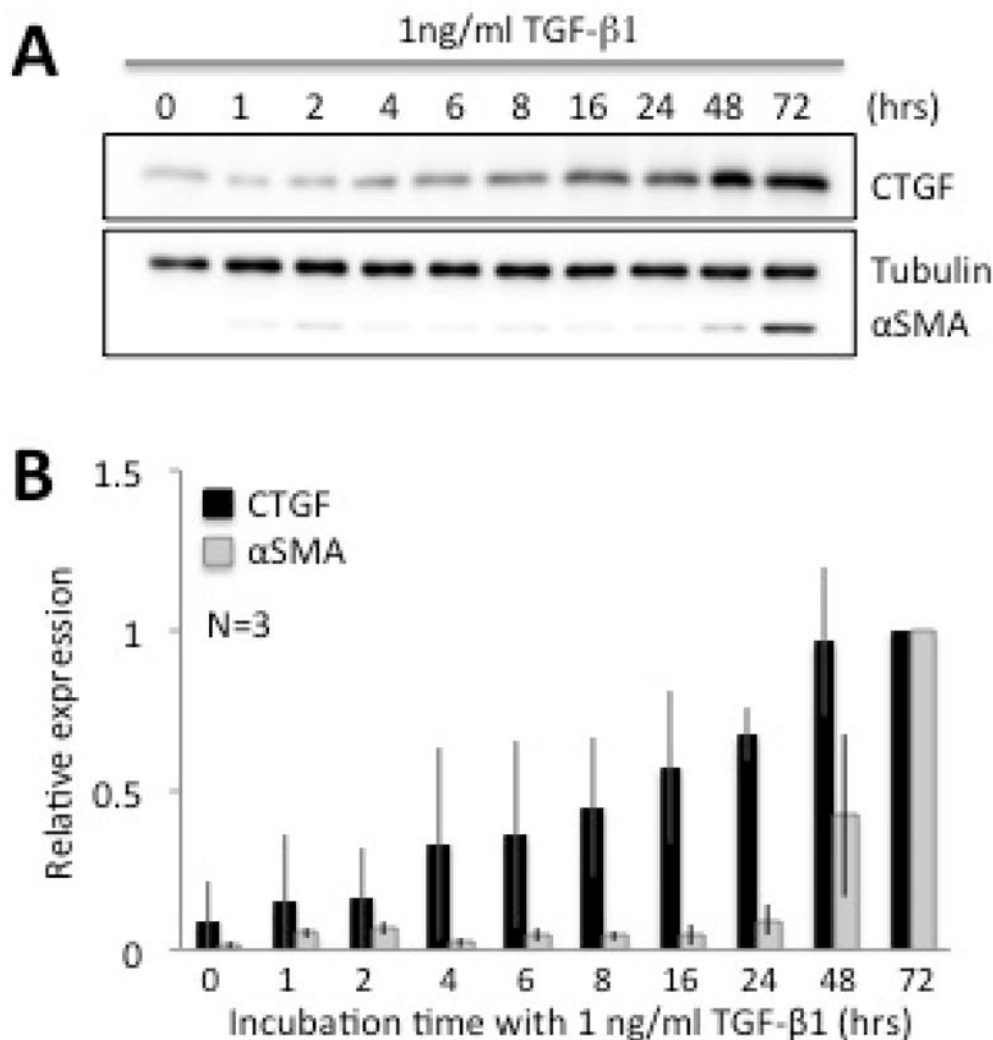


Figure 1. Time-dependent TGF- β -induced expression of CTGF and α SMA in cultured cat corneal fibroblasts

A. Representative western blots showing protein levels for CTGF and α SMA at different time-points (in hours) after TGF- β 1 stimulation. β -Tubulin levels were assayed as a loading control. The basal level of CTGF was distinctly above zero. After 4 hours of culture, TGF- β 1 started to affect the expression of CTGF, while α SMA expression remained too low to assay reliably until 48 hrs after stimulation. CTGF protein expression appeared to reach maximal levels between 48 and 72 hrs, while α SMA expression was greatest at 72 hrs, as previously reported for this cell culture system (Jeon et al 2014). β -Tubulin levels remained stable throughout. **B.** Plots of relative expression of CTGF and α SMA with respect to Tubulin expression at different time points after stimulation with 1ng/ml TGF- β . Values were normalized to the maximal levels attained (at 72 hrs for both CTGF and α SMA). Data shown are means \pm SD, averaged over 3 experiments.

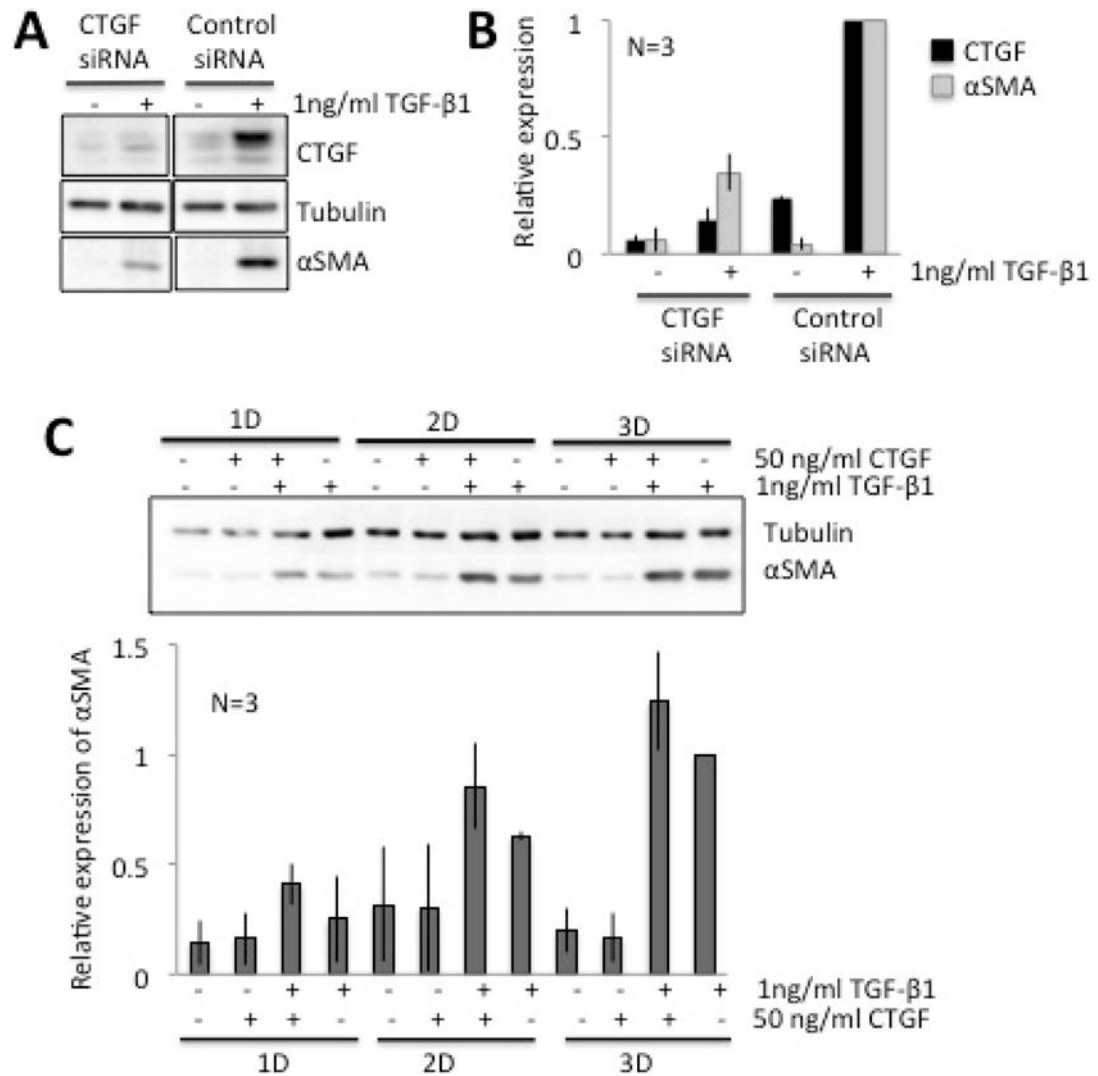


Figure 2. Role of CTGF in TGF-β-induced corneal myofibroblast differentiation

A. Representative Western blot showing protein levels for CTGF and αSMA in cells transfected with either CTGF siRNA or control (non-targeting) siRNA. β-Tubulin levels were assayed as a loading control. CTGF siRNA blocked the TGF-β-induced increases in CTGF and αSMA levels compared with control siRNA. β-tubulin levels remained stable throughout. **B.** Plot of relative densitometry data for CTGF and αSMA expression with respect to Tubulin, which was further normalized to values obtained in cells stimulated with 1ng/ml TGF-β following transfection with either CTGF or control siRNA (see sample blot in **B**). **C.** Representative Western blot showing protein levels for αSMA in cells treated with C-terminus, recombinant human CTGF with/without TGF-β. β-Tubulin levels were assayed as a loading control. Exogenously added CTGF potentiated the TGF-β-induced increase in αSMA levels compared with TGF-β alone, especially after 2 and 3 days in culture. β-tubulin levels remained stable throughout. The graph below is a plot of αSMA expression relative to β-tubulin expression, normalized to values obtained in cells stimulated with 1ng/ml TGF-β for 3 days. Data shown are means ± SD over 3 experiments.

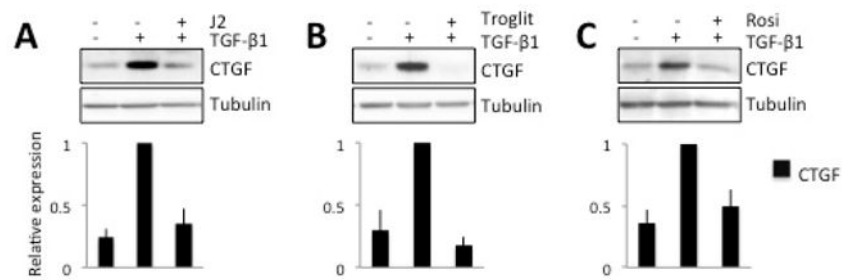


Figure 3. Effect of PPAR γ ligands on TGF- β 1-induced CTGF expression in cultured corneal fibroblasts

A. Representative Western blots showing protein levels for CTGF and β -tubulin, which was assayed as a loading control. After 1 day in culture, 5 μ M 15d-PGJ2 (J2) blocked the TGF- β -induced increase in CTGF expression, while β -tubulin levels remained stable. These effects are confirmed in the plot of relative expression of CTGF normalized to densitometric values obtained in cells stimulated with 1 ng/ml TGF- β . **B.** Representative blot illustrating the impact of 15 μ M Troglitazone (T), which also effectively blocked the TGF- β -induced increase in CTGF expression. The densitometry plot below confirms this result over 3 separate experiments. **C.** Representative blot illustrating the impact of 75 μ M Rosiglitazone (R) on the TGF- β -induced increase in CTGF expression after 1 day in culture. As in **A** and **B**, β -tubulin levels were unaffected. These effects are confirmed in the densitometry plot showing decreased relative expression of CTGF compared to the TGF- β -stimulated condition. In all cases, data shown are means \pm SD, averaged over 3 experiments.

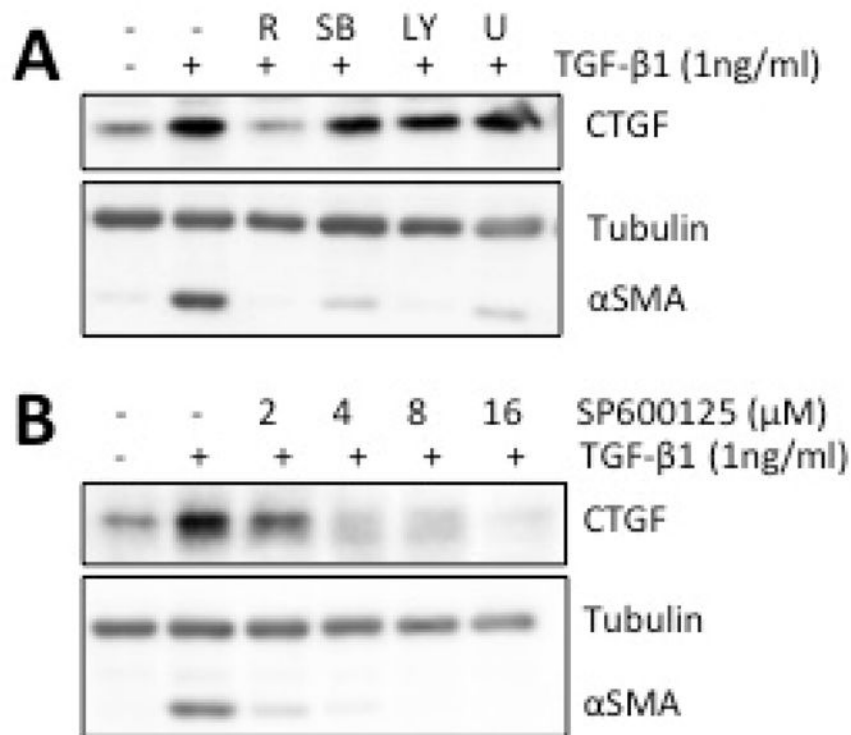


Figure 4. Effect of kinase inhibitors on TGF-β-induced expression of CTGF and αSMA

A. Representative Western blots showing the effect of incubating cultures with the PPAR γ ligand Rosiglitazone (R, 75 μ M) or different kinase inhibitors [for ERK: 10 μ M U0126 (U), for p38 MAPK: 6 μ M SB203580 (SB) and for AKT: 5 μ M LY294002 (LY)]. While Rosiglitazone and all 3 inhibitors blocked TGF- β -induced increases in the expression of α SMA, only Rosiglitazone effectively blocked CTGF induction. β -Tubulin levels were assayed as a loading control and remained unaffected by the different treatments. **B.** Representative Western blots showing the effect of different doses of the JNK inhibitor (SP600125) on TGF- β -induced increases in the expression of CTGF and α SMA in cultured corneal fibroblasts. β -Tubulin levels were assayed as a loading control. SP600125 blocked the expression of both CTGF and α SMA in a dose-dependent manner, although it appeared to inhibit α SMA at a lower dose than CTGF.

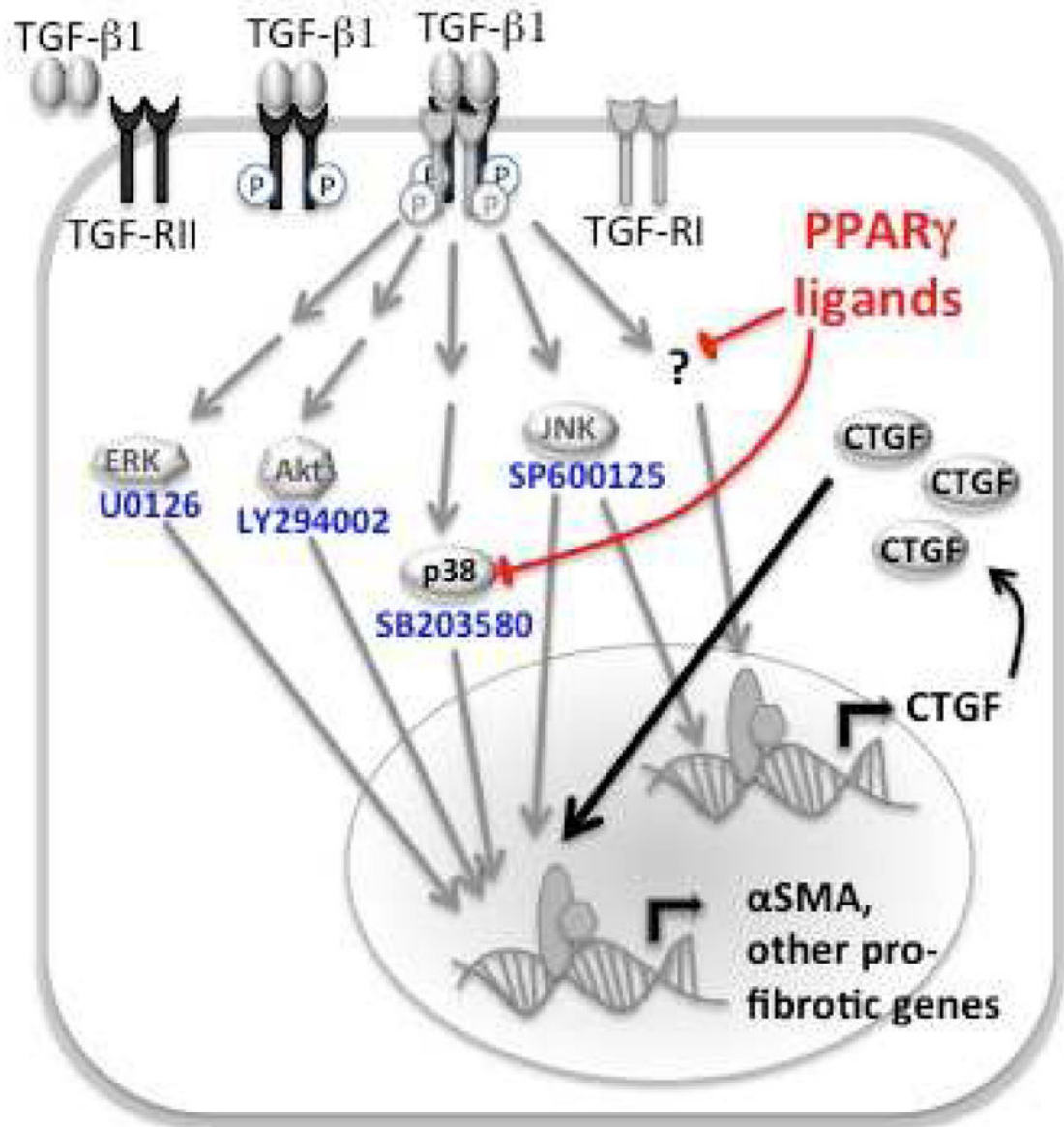


Figure 5. Schematic diagram of pro-fibrotic signaling pathways activated by TGF- β binding to its receptor in corneal fibroblasts
 TGF- β 1 increases α SMA expression through activation of ERK, Akt, p38, JNK and CTGF. PPAR γ ligands block TGF- β 1-induced up-regulation of CTGF, independently of p38 MAPK in corneal fibroblasts