MOLECULAR MECHANISMS OF TGF-β/SMAD SIGNALING CASCADE INDUCED BY CYCLOSPORIN A IN A549 LUNG EPITHELIAL CELLS

BY

El-Sayed Akool

FROM

Pharmacology and Toxicology Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt

ABSTRACT

Transforming growth factor-β (TGF-β) and downstream Smad signaling pathways have been found to be the most important pathways involved in tissue fibrosis via induction of the profibrotic genes connective tissue growth factor (CTGF) and tissue inhibitors of matrix metalloproteinase-1 (TIMP-1). Cyclosporin A (CsA) is one of the most efficient immunosuppressive drugs that has been shown to induce a rapid activation of TGF-β/Smad signaling pathway. The present work demonstrates that CsA similar to TGF-β causes a rapid phosphorylation of Smad-2 in A549 lung epithelial cells which is abrogated by the addition of neutralizing TGF-β-antibody. By using the TGF-β receptor I kinase inhibitor, activin receptor-like kinase-5 (ALK-5) a critical involvement of TGF-β receptor in CsA-triggered Smad signaling was depicted. Furthermore, TGF-β release and Smad-2 phosphorylation induced by CsA were highly reduced in the presence of the reactive oxygen species (ROS) scavenger N-acetyl-cysteine (NAC), indicating that ROS is required for TGF-β release and Smad-2 phosphorylation induced by CsA. Moreover, the present work demonstrates that CsA via ROS generation and activation of TGF-β/Smad signaling cascade can cause an increase in the expression of the profibrotic genes CTGF and TIMP-1 in A549 lung epithelial cells. Collectively, these data demonstrate that CsA causes a rapid activation of the fibrogenic Smad signaling cascade in A549 lung epithelial cells via generation of ROS and subsequent activation of latent TGFβ that is sufficient to elicit fibrogenic cell responses as indicated by increasing the expression of the fibrogenic genes CTGF and TIMP-1 that play an important role in tissue fibrosis.

INTRODUCTION

Fibrotic diseases have been shown to be associated with excessive deposition of extracellular matrix (ECM) (Wells, 2008; Cox & Erler, 2011; Klingberg et al., 2013). The accumulation of ECM is thought to be primarily due to an impaired ECM turnover (Duymelinck et al., 1998). ECM degradation is mainly regulated by the action of two matrix-degrading enzyme systems, the matrix metalloproteinases and the plasminogen activators and their intrinsic inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) and the plasminogen activator inhibitors, respectively (Woessner, 1991; Eddy, 2000). Transforming growth factor-β (TGF-β) and downstream Smad signaling pathways have been found to be the most important pathways involved in tissue fibrosis via induction of profibrotic genes (Roberts & Sporn, 1990; Gore-Hyer et al., 2002; Ruiz et al., 2003). Generally, TGF-

β induces ECM deposition by stimulating the expression of ECM proteins such as connective tissue growth factor (CTGF), reducing synthesis of ECM degrading enzymes such as matrix metalloproteinases-9 (MMP-9) and up-regulating synthesis of proteinase inhibitors such as tissue inhibitors of metalloproteinase-1 (TIMP-1) (Roberts & Sporn, 1990; Gore-Hyer et al., 2002; Ruiz et al., 2003; Weng et al., 2007; Wang et al., 2011). TGF-β is usually secreted as latent complex (latent TGF-β) consisting of TGF-β covalently bound to latent TGF-β binding proteins (LTBP) (Roberts, 1998; Okada et al., 2005). Activation of TGF-β is achieved by either proteolytic or nonproteolytic events (Annes et al., 2003) and in some cases may include a redox-sensitive mechanism (Barcellos-Hoff & Dix, 1996; Jobling et al., 2006). In this respect, oxidation of the latency-associated peptide can cause a conformational change that releases TGF-β (Barcellos-Hoff & Dix, 1996). Mechanistically, activated TGF-β acts through a heteromeric receptor complex. The predominant type 1 receptor for TGF-β signaling is thought to be activin receptor-like kinase (ALK)-5 which phosphorylates Smad-2 and Smad-3 and subsequently forms a complex with the co-Smad, Smad-4, and translocates into the nucleus. Subsequently, these complexes bind with a high affinity to specific promoter elements, the Smad binding elements (SBE), and thereby can activate the transcription of many TGF-β-induced target genes, including CTGF (Fukasawa et al., 2004) and TIMP-1 (Marti et al., 1994; Chen et al., 2002). Cyclosporin A (CsA) is one of the most efficient immunosuppressive agents that suppress T-cell activation by inhibiting the cellular phosphatase calcineurin (Schreiber & Crabtree, 1992) and therefore is widely used in organ transplantation and many inflammatory diseases including psoriasis, and rheumatoid arthritis. Recently, CsA has been shown to cause a rapid activation of TGF-β/Smad signaling pathway in glomerular mesangial cells and thereby increase the expression of the profibrotic genes CTGF and TIMP-1 (Akool et al., 2005 and 2008). The present work was designed to test the possible modulation of TGF-β/Smad signaling pathway by CsA in pulmonary epithelial cells (A549 cells) that are considered as a source of TGF-β in the lung (Kwong et al., 2004).

MATERIALS AND METHODS

Materials

The TGFβ-RI kinase inhibitor [3-(pyridin-2-yl)-4-(4-quinonyl)]-1*H*-pyrazole (Alk-5 inhibitor), U0126, SP600125 and SB203580 were obtained from Calbiochem (Germany). Diphenylene iodonium (DPI), *N*-acetyl cysteine (NAC) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Human recombinant $TGF\beta_1$ was purchased from Cell Concepts GmbH (Umkirch, Germany). CsA was purchased from Alexis Biochemicals (Germany). A neutralizing monoclonal $TGF\beta_{1-3}$ antibody and Human TGF-β_1 Quantikin-e ELISA Kit were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). Antibodies specifically raised against phospho-Smad-2, total Smad-2 were derived from Cell Signaling (Frankfurt am Main, Germany). Antibodies against CTGF, TIMP-1, β-actin and anti-rabbit HRPlinked IgGs, as well as control IgG were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The ECL system, and Hyperfilm were purchased from Amersham Pharmacia Biotech (Germany).

Cell Culture

Human A549 lung epithelial/carcinoma cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were cultivated in RPMI 1640, supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 10% heat-inactivated FCS and 10mM HEPES buffer (GIBCOBRL, Eggenstein, Germany).

Western Blot Analysis

For detection of CTGF, TIMP-1, phosphorylated Smad-2 and total Smad-2, whole-cell lysates were prepared. Total cell extracts containing 50-100 µg of protein were prepared in sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis was performed as originally described by Laemmli (1970). In general, a total protein (50-100µg) from each sample was mixed with an equal volume of 2x electrophoresis sample buffer and incubated at 95°C for 10 minutes for denaturation. Following gel electrophoresis, the proteins were transferred onto a nitrocellulose (PVDF) membrane by semi-dry electroblotting. After blocking (by shaking the membrane in 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.05% Tween for 1 hour, the membrane was incubated with the primary antibody overnight at 4°C followed by incubation with secondary antibodies (coupled to horseradish peroxidase). Signals were detected using enhanced chemi luminescence (ECL) reagent according to the manufacturer's instructions.

ELISA

Levels of TGF-β in cell-free culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Wiesbaden-Nordenstadt, Germany).

Statistical Analysis

Results are expressed as means ± SD. Statistical analysis was performed using Student's *t* test and for multiple comparisons the ANOVA test for significance. *P*-values below 0.05 were considered as indication for statistically significant differences between conditions compared.

RESULTS

Cyclosporin A rapidly activates Smad-2 in A549 cells

First to test whether CsA similar to TGF-β could induce phosphorylation of Smad-2 a direct target of the activated type I TGF-β receptor. A549 cells were stimulated with either vehicle (control) or CsA or TGf-β for different time points. Stimulation of A549 cells with CsA induced a rapid phosphorylation of Smad-2 with maximal effect seen after 60 min (Fig.1A). This was not associated with change in the total Smad-2 content. TGF-β, a potent activator of Smads was used as a positive control. As expected, TGF-β_1 caused a strong increase in Smad-2 phosphorylation (Fig. 1A).

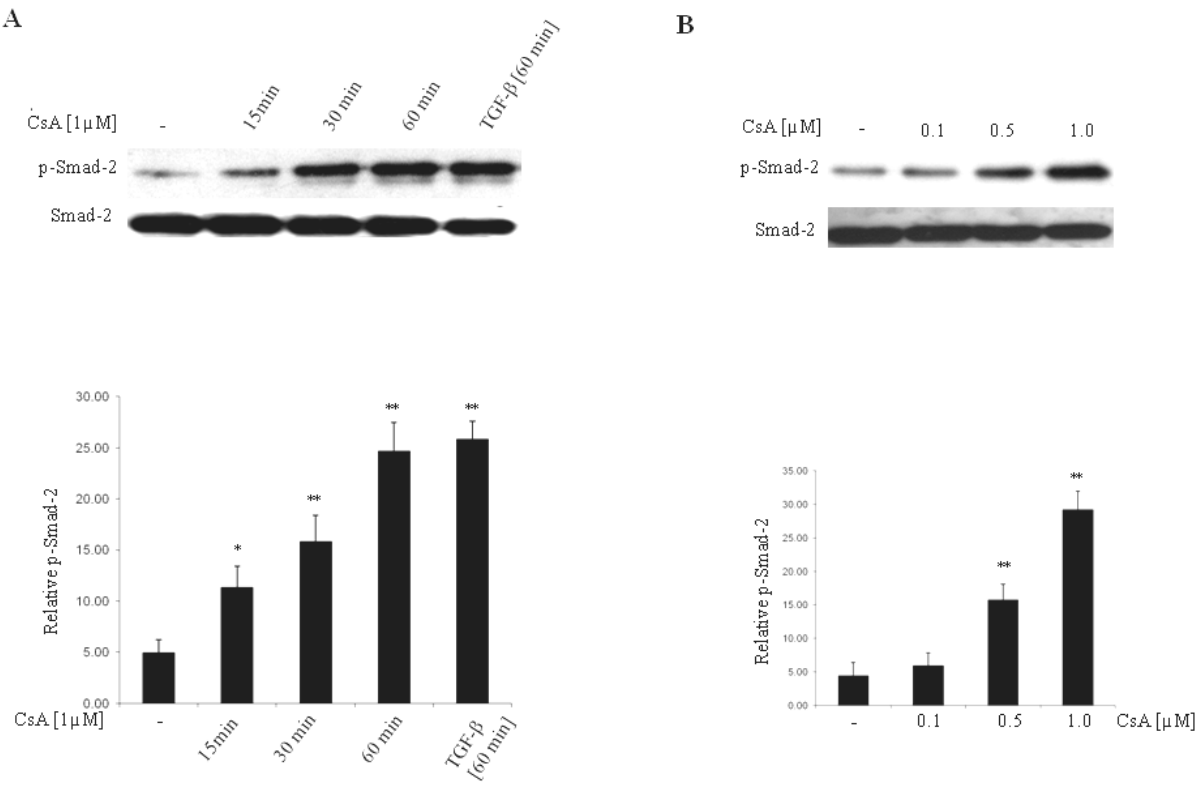

Figure 1. CsA similar to TGF-β activates Smad-2 in A549 cells. A, A549 cells were stimulated with either vehicle (-) for 60 min or with TGF-β (10ng/ml) or CsA (1µM) for the indicated time points. Thereafter, total extracts were isolated and Western blot analysis was performed using specific anti-phospho-Smad-2 at a dilution of 1:1000 each. For ascertainment that the total level of Smad-2 remained unchanged, blots were stripped and reprobed with the antibody raised against total Smad-2. **B**, A549 cells were stimulated with either vehicle for 60 min. or different doses of CsA (µM) for 60 min. Thereafter, total extracts were isolated and Western blot analysis was performed as previously described. The lower panels show a densitometric analysis of p-Smad-2 relative to total Smad-2 level. Data represent means ± S.D. (n=3), * $p < 0.05$, ** $p < 0.01$ versus control. One representative of three independently performed experiments is shown.

CsA Activates Smad-2 in dose-dependent manner

To check the effect of different doses of CsA on Smad-2 activation, A549 cells were stimulated with different doses of CsA. As shown in Fig.1B CsA activates Smad-2 in a dose-dependent manner with maximal effect seen at a dose of 1µM.

Activation *of Smad-2 by CsA in A549 cells depends on extracellular TGF-β and involves TGF-β-RI kinase*

To delineate the mechanism by which CsA activates Smad-2, the involvement of extracellular TGF-β as well as TGF-β-RI kinase in Smad-2 phosphorylation induced by CsA was tested using pan-specific TGF-β antibody and the specific-TGF-β-RI kinase inhibitor which has demonstrated a high TGF-β-inhibitory potential in vitro and in vivo. A549 cells were preincubated without (+ vehicle) or with either 20µg/ml of pan- specific TGF-β antibody (+ anti-TGFβ a.b.) or, alternatively, with the same amount of control IgG (+ IgG) or 100nM of TGF-β-RI kinase inhibitor before cells were additionally treated with

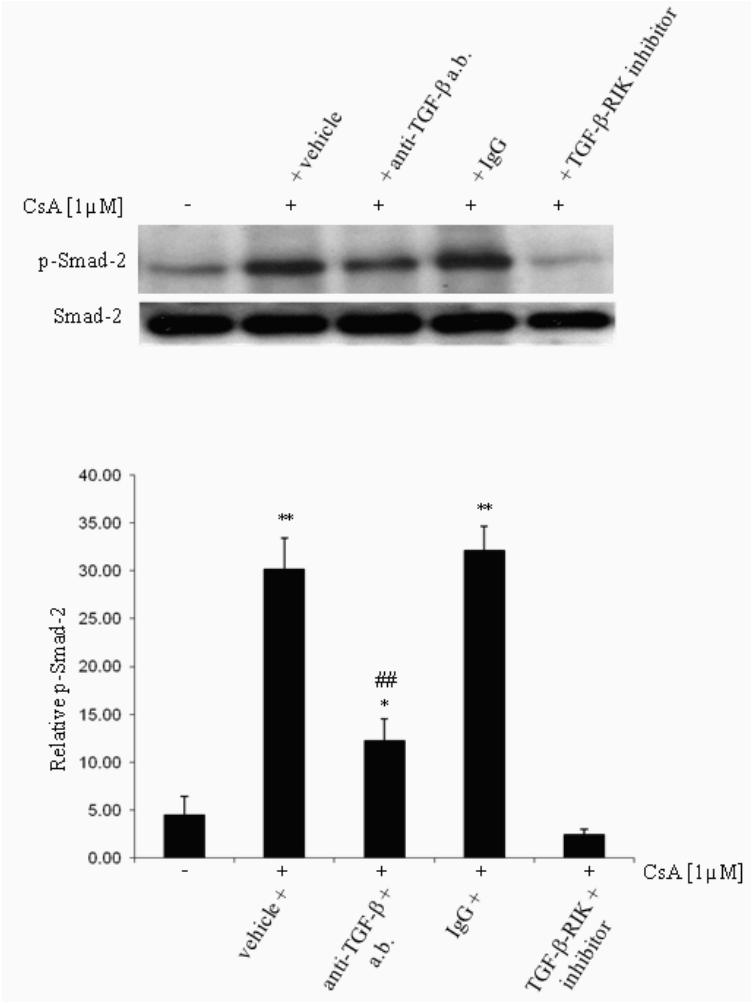

Figure 2. CsA-triggered Smad-2 phosphorylation depends on extracellular TGF-β and TGF-βRI kinase activity in A549 cells. A549 cells were pretreated for 60 min with either vehicle or pan-specific TGF-β antibody (20μg/ml) or the same amount of control IgG or TGF-β RI kinase inhibitor (100nM) before cells were stimulated for a further 60 min with either vehicle (-) or CsA. Thereafter, total extracts were isolated and Western blots successively probed with anti-phospho-Smad-2. For ascertainment that the total level of Smad-2 remained unchanged, blots were stripped and reprobed with the antibody raised against total Smad-2. The lower panel shows a densitometric analysis of p-Smad-2 relative to total Smad-2 level. Data represent means ± S.D. (n=3), * $p < 0.05$, ** $p < 0.01$ versus control, ## $p < 0.01$ versus CsA alone-treated animals. One representative of three independently performed experiments is shown.

CsA for 60min (Fig. 2). The neutralization of TGF-β caused a significant reduction in CsA-induced Smad-2 phosphorylation, whereas addition of isotype-specific control IgG (+IgG) had no effect on p- Smad-2 levels (Fig. 2). Furthermore, it was found that preincubation of A549 cells with 100 nM of the TGF-β-RI kinase inhibitor totally prevented basal as well as CsA-dependent Smad-2 phosphorylation (Fig. 2).

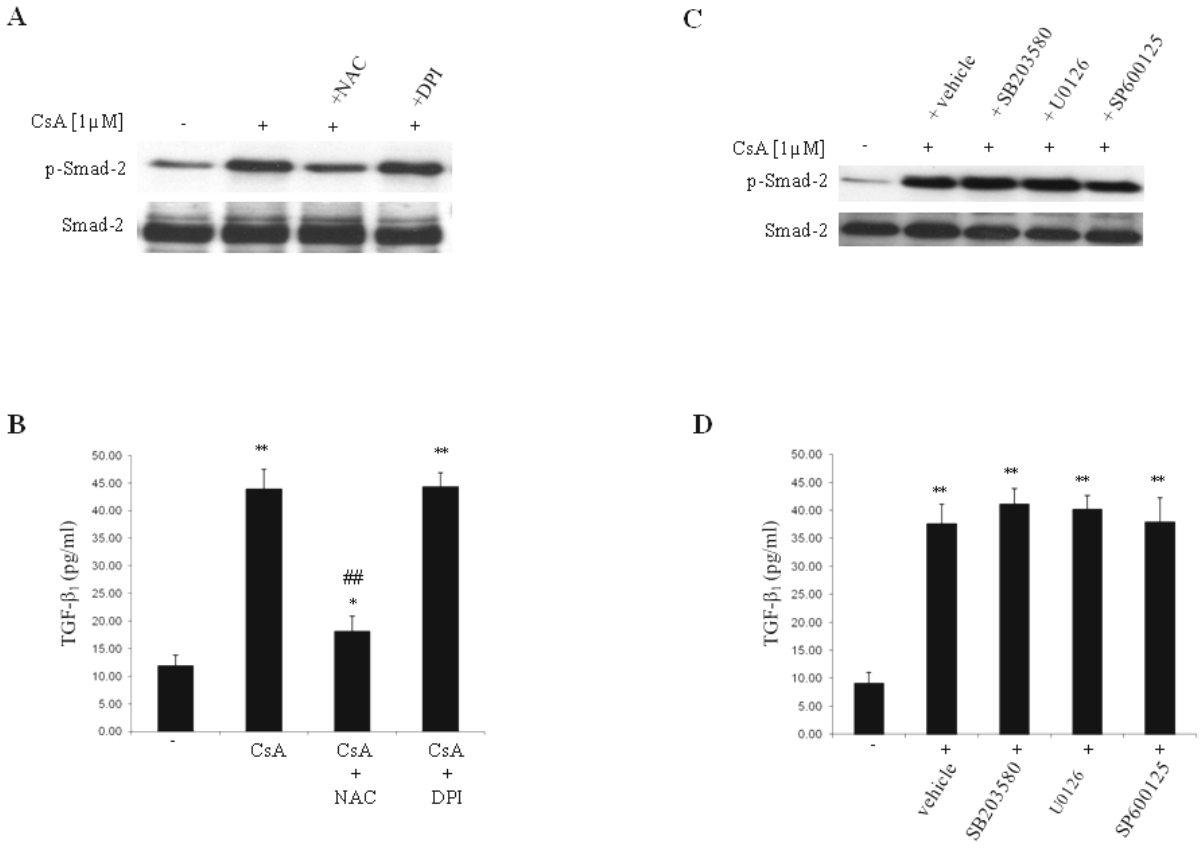

Figure 3. TGF-β release and Smad-2 phosphorylation induced by CsA in A549 cells depends on ROS but not MAPKs. A, B. A549 cells were preincubated with NAC (5mM) or DPI (10µM) for I hour before stimulation with CsA for further hour. After stimulation with CsA, Cell supernatants were collected and assessed for active TGF-β_1 by Quantikine ELISA (**B**). Thereafter, total extracts were isolated and Western blots successively probed with anti-phospho-Smad-2. For ascertainment that the total level of Smad-2 remained unchanged, blots were stripped and reprobed with the antibody raised against total Smad-2 (**A**). **C, D.** A549 cells were stimulated for 60 min with CsA in the absence or presence of different MAPK inhibitors, SB203580 (10µM), U0126 (20 µM), SP600125 (10µM), which were preincubated for 60 min. After stimulation with CsA, Cell supernatants were collected and assessed for active TGF-β_1 by Quantikine ELISA (**D**). Thereafter, total extracts were isolated and Western blot analysis was performed as previously described (**C**). Data represent means ± S.D. (n=3), * $p < 0.05$, ** $p < 0.01$ versus control, ## $p < 0.01$ versus CsA alone-treated animals. One representative of three independently performed experiments is shown.

ROS but not MAPKs are required for TGF-β activation and subsequent smad-2 phosphorylation by CsA in A549 cells

To check the involvement of ROS in Smad-2 activation by CsA, A549 cells were preincubated with either NAC a ROS scavenger or DPI, an inhibitor of NADPH oxidases for 1 hour before stimulation with CsA for further hour. In contrast to DPI, NAC caused a significant inhibition of CsA-induced Smad-2 activation (Fig. 3A). Interestingly, this inhibitory effect of NAC on CsA-induced Smad-2 phosphorylation was associated with reduction in TGF-β released by CsA (Fig. 3B). The involvement of MAPK activity in Smad-2 phosphorylation by CsA was also investigated. As shown in Fig. 3C, neither p38 MAPK inhibitor (SB203580) nor p44/42 MAPK inhibitor (U0126) nor JNK inhibitor (SP600125)

were able to inhibit Smad-2 phosphorylation induced by CsA. Furthermore, the release of TGF-β by CsA was not affected in the presence of MAPK inhibitors (Fig. 3D).

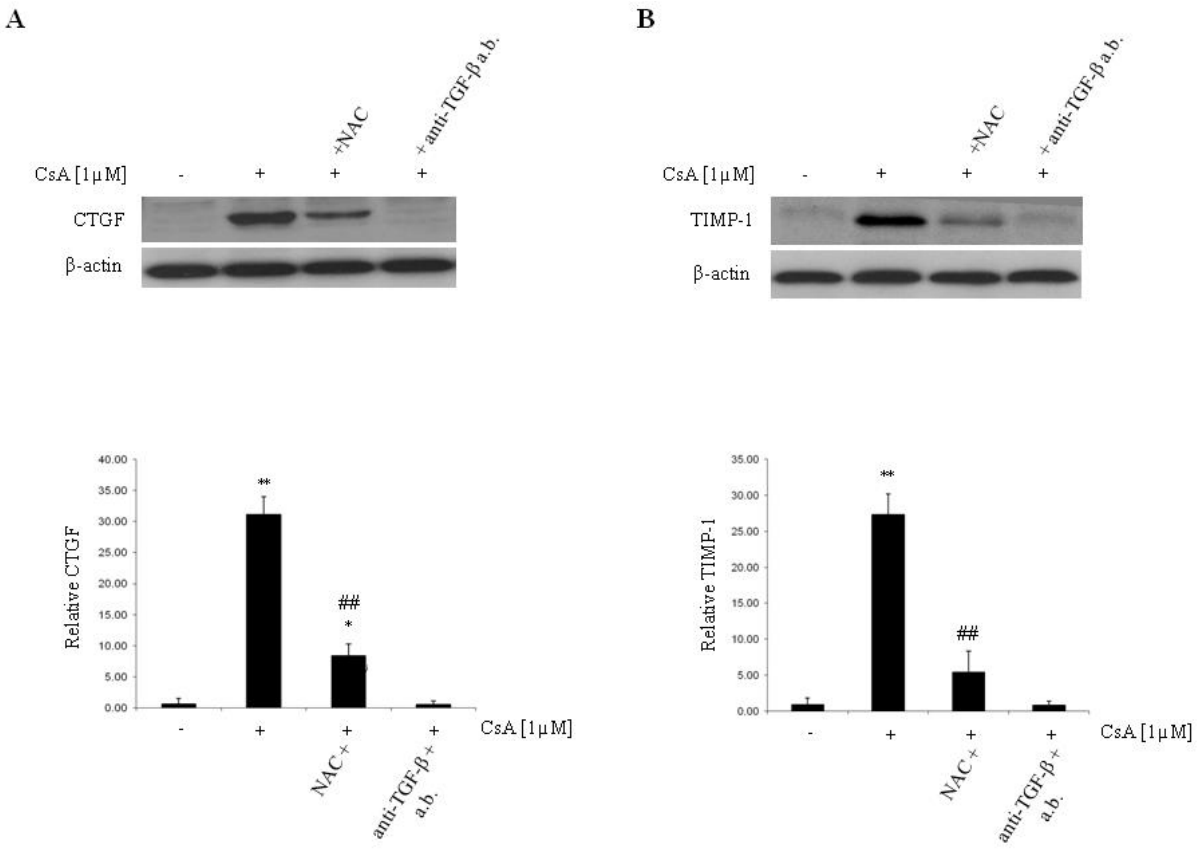

Figure 4. CTGF and TIMP-1 expression induced by CsA in A549 cells depends on ROS and extracellular TGF-β. A549 cells were stimulated for 16 h with CsA (1μM) in the absence or presence of NAC (5mM) or 20μg/ml of a pan-specific TGF-β antibody. Thereafter, cells were harvested for total protein extracts and Western blots were probed with anti-CTGF and anti-TIMP-1. Loading of equal amounts of total extracts was ascertained by reprobing the blots with β-actin antibody. The lower panels show a densitometric analysis of CTGF and TIMP-1 relative to β-actin level. Data represent means ± S.D. (n=3), * $p < 0.05$, ** $p < 0.01$ versus control, ## $p < 0.01$ versus CsA alone-treated animals. One representative of three independently performed experiments is shown.

ROS and TGF-β are required for CTGF and TIMP-1 expression induced by CsA in A549 cells

As shown in Fig.4, stimulation of A549 cells with CsA increased the expression of CTGF (Fig. 4A) and TIMP-1(Fig. 4B). However, preincubation with a neutralizing pan-specific TGF-β antibody (+ anti-TGFβ a.b) caused a strong reduction in CsA-induced expression of CTGF (Fig. 4A) and TIMP-1 (Fig. 4B). Furthermore, preincubation with NAC caused a clear reduction in CsA-induced expression of CTGF (Fig. 4A) and TIMP-1 (Fig. 4B).

DISCUSSION

TGF-β and downstream Smad signaling pathways have been found to be the most important pathways involved in tissue fibrosis by increasing the expression of the profibrotic genes CTGF and TIMP-1 (Roberts & Sporn, 1990; Gore-Hyer et al., 2002; Ruiz et al., 2003; Weng et al., 2007; Wang et al., 2011). It has been reported that the profibrotic actions of the immunosuppressive drug CsA is attributable to an autocrine synthesis of TGF-β (Wolf et al., 1993; Pankewycz et al., 1996; Khanna et al., 1999), and some studies have demonstrated a transcriptional effect on TGF-β expression by CsA both in vitro (Prashar et al., 1995) and in vivo (Citterio et al., 2004; Gao et al., 2006). Recently, it has been demonstrated that CsA causes a rapid activation of TGF-β/Smad signaling cascades in renal mesangial cells (Akool et al., 2008) and thereby increase the expression of the profibrotic genes CTGF and TIMP-1 (Akool et al., 2005 and 2008). The present work was designed to test the possible modulation of TGF-β/Smad signaling pathway by CsA in pulmonary epithelial cells (A549 cells) that are considered as a source of TGF-β in the lung (Kwong et al., 2004). It was found that treatment of A549 cells with CsA caused a rapid phosphorylation of Smad-2 in dose-and time-dependent manner (Fig. 1). To delinate the underlying mechanisms involved in Smad signaling activation by CsA, A549 cells were treated with a specific kinase inhibitor of the type I TGF-β receptor denominated as ALK-5 inhibitor, and a neutralizing TGF-β antibody before stimulation with CsA. It was observed that TGF-β receptors and TGF-β release are indispensable for Smad-2 activation by CsA (Fig. 2). Mechanistically, the release of TGF-β from the latent TGF-β binding protein (LTBP) complex can be achieved by either proteolytic or nonproteolytic events (Annes et al., 2003.) and in some cases may include a redox-sensitive mechanism (Barcellos-Hoff & Dix, 1996; Jobling et al., 2006). In this context, oxidation of the latency conferring peptide can cause a conformational change that releases TGFβ (Barcellos-Hoff & Dix, 1996). Previous studies have demonstrated that CsA at different concentrations promotes the generation of ROS by a mechanism that is independent of cytochrome P-450 oxidases (Krauskopf et al., 2002) and NADPH oxidases (Krauskopf et al., 2005), the major cellular superoxide-generating enzymes. In full agreement with these studies, It was found that preincubation of A549 cells with the ROS scavenger N-acetylcysteine (NAC) before stimulation with CsA highly reduced the Smad-2 activation (Fig. 3A). In contrast, nothing changed with Smad-2 activation when the cells were preincubated with DPI (NADPH oxidase inhibitor) before stimulation with CsA indicating that NADPH oxidase is not the source of ROS in A549 cells (Fig. 3A). Most interestingly, this inhibitory effect of NAC on CsA-induced Smad-2 phosphorylation was associated with reduction in TGF-β released by CsA (Fig. 3B) indicating that ROS generation is required for TGF-β release and subsequent Smad-2 phosphorylation induced by CsA in A549 cells. MAPKs have been implicated in Smads signaling (Javelaud & Mauviel, 2005; Akool et al., 2008). Therefore, the involvement of MAPK activity in Smad-2 phosphorylation was tested**.** In contrast to previous finding but in other system (Akool et al., 2008), it was found that MAPKs are not involved in Smad-2 phosphorylation induced by CsA in A549 cells (Fig. 3C). Furthermore, the release of TGF-β induced by CsA was not affected in the presence of

MAPK inhibitors (Fig. 3D) indicating that MAPK activity is not involved in TGF-β release as well as Smad-2 phosphorylation induced by CsA. To test whether the Smad-2 activation induced by CsA would functionally correlate with an up-regulation of Smad controlled gene expression, CTGF and TIMP-1 expression were investigated. In agreement with previous findings but in other system (Akool et al., 2008), treatment of A549 cells with CsA induces CTGF (Fig. 4A) and TIMP-1 (Fig. 4B) expression. Most interestingly, preincubation of the cells with a neutralizing TGF-β antibody or NAC before stimulation with CsA caused a strong reduction in CsA-induced expression of CTGF (Fig. 4A) and TIMP-1 (Fig. 4B) indicating that ROS and TGF-β are required for the induction of CTGF and TIMP-1 expression by CsA in A549 cells. In summary, CsA causes a rapid activation of the fibrogenic Smad signaling cascade in A549 cells via generation of ROS and subsequent activation of latent TGFβ that is sufficient to elicit fibrogenic cell responses as indicated by increasing the expression of the fibrogenic genes CTGF and TIMP-1 that play an important role in tissue fibrosis. The potential blockade of TGF-β/Smad signaling pathway, either by anti-TGFβ Abs or antioxidant treatment, may emphasize the concept of therapeutic TGFβ neutralization in combination with antioxidant therapy as a valuable approach for the prevention of CsA-induced profibrotic genes.

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الميكانيكية الجزيئية لتحفيز إشارات عامل النمو بيتا- سماد فى خلايا الرئة الظهارية (A549) بواسطة مثبط المناعة السيكلوسبورين أ

للسيد الدكتور

السيد عاقول

مـــــــن

قسم علم الأدويه والسموم- كلية الصيدلة- جامعة الأزهر

يعتبر عامل النمو بيتا من اهم العوامل التى تلعب دوراً هاماً فى تليف الانسجة, وذلك من خلال تنشيط إشارات سماد التى تؤدى الى زيادة انتاج عامل نمو الانسجة الضامة (CTGF) وكذلك مثبط الماتريكس ميتالوبروتينيز-١ (TIMP-1)واللذان يلعبان دوراً هاماً فى تليف الانسجة .

الهدف من هذه الدراسة هو معرفة ما اذا كان السيكلوسبورين أ (المثبط للمناعة) يمكنه تحفيز إشارات عامل النمو بيتا- سماد فى خلايا الرئة الظاهرية (A549). كما استهدفت الدراسة ايضاً معرفة الميكانيكية الجزيئية لتحفيز هذه الاشارات بواسطة مثبط المناعة السيكلوسبورين أ.

وقد اتضح من خلال هذا البحث أن مثبط المناعة السيكلوسبورين أ ينشط اشارات عامل النمو بيتا- سماد فى خلايا الرئة الظاهرية (A549). وذلك عن طريق انتاج فصائل الاكسجين الحرة , حيث انه قد لوحظ أن استخدام المواد المضادة للاكسدة تقلل الى حد كبير جداً من نشاط عامل النمو وكذلك اشارات سماد الناتجة عنه. كما لوحظ ايضاً ان معادلة عامل النمو بيتا النشط باستخدام اجسام مضادة له تقلل الى حد كبير جداً من اشارات سماد مما يوضح أن السيكلوسبورين أ يحفز اشارات عامل النمو بيتا- سماد من خلال انتاج فصائل الاكسجين الحرة التى تنشط عامل النمو بيتا والذى بدوره يحفز اشارات سماد كما وجد ايضا أن اشارات عامل النمو بيتا- سماد تؤدى فى النهاية الى انتاج عامل نمو الانسجة الضامة (CTGF) وكذلك مثبط الماتركس ميتالوبروتينيز-١(TIMP-1) واللذان يلعبان دوراً هاماً فى تليف الانسجة.

كما اتضح ايضاً من هذه الدراسة أن إنتاج عامل نمو الانسجة الضامة (CTGF) وكذلك مثبط الماتركس ميتالوبروتينيز-١ (TIMP-1) يعتمد على فصائل الاكسجين الحرة وعامل النمو بيتا.

مما سبق يتضح ان السيكلوسبورين أ من خلال انتاجه لفصائل الاكسجين الحرة يستطيع تنشيط اشارات عامل النمو بيتا- سماد فى خلايا الرئة الظاهرية (A549) والتى ينتج عنها زيادة فى انتاج عامل نمو الانسجة الضامة (CTGF) وكذلك مثبط الماتركس ميتالوبروتينيز-١(TIMP-1) واللذان يلعبان دوراً هاماً فى تليف الانسجة.