CYCLOSPORIN A ACTIVATES TGF-β/SMAD SIGNALING PATHWAY IN RAT LIVER

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ABSTRACT

Cyclosporin A (CsA) is one of the most important immunosuppressive agents and therefore is widely used in organ transplantation. However, the clinical use of CsA is strongly limited by several side effects including hepatotoxicity which remains a major clinical problem. Transforming growth factor-β (TGF-β) and downstream Smad signaling pathways have been found to play an important role in liver fibrosis via induction of profibrotic genes such as tissue inhibitors of matrix metallproteinases-1 (TIMP-1), and connective tissue growth factor (CTGF). The present work demonstrates that treatment of animals with CsA causes a rapid activation of TGF-β/Smad signaling cascade in rat liver as demonstrated by an increase in plasma TGFβ level and Smad-2 phosphorylation. Activation of TGF-β/Smad signaling cascade was accompanied by activation of Smad-dependent expression of TIMP-1and CTGF. However, concomitant administration of neutralizing anti-TGF-β antibody markedly reduced Smad-2 phosphorylation as well as CTGF and TIMP-1 expression induced by CsA. Furthermore, it was found that administration of the antioxidant N-acetyl cysteine (NAC) along with CsA significantly reduced plasma TGF-β level, Smad-2 phosphorylation as well as CTGF and TIMP-1 expression. These data demonstrates for the first time that administration of CsA causes a rapid activation of TGF-β/Smad signaling pathway and subsequent CTGF and TIMP-1 expression in rat liver.

INTRODUCTION

Solid organ transplantation has become one of the most important fields that play an important role in decreasing the mortality rate of patients with organs failure. However, the risk of organ rejection threatens the success of the transplantation procedure. The reaction of the immune system to the transplanted organ can be prevented by the use of an immunosuppressive agent. 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. However, the clinical use of CsA is strongly limited by several side effects including liver toxicity which remains a major clinical problem (Durak et al., 2004; Hagar, 2004; Rezzani, 2006; Kaya et al., 2008). Liver fibrosis is a major

cause of hepatic failure. It is characterized by excessive accumulation of extracellular matrix (ECM) (Biagini & Ballardini, 1989; Wells, 2008). Connective tissue growth factor (CTGF) and tissue inhibitors of metalloproteinases (TIMP-1) play an important role in the pathogenesis of liver fibrosis by regulating the synthesis and degradation of ECM (Arthur et al., 1998; Weng et al., 2007). Recently, it has been reported that CTGF and TIMP-1 may be effective targets for therapeutic intervention of liver fibrosis (Wang et al., 2013; Rachfal, & Brigstock, 2003; Lipson et al., 2012). The expression of CTGF and TIMP-1 is regulated by various stimuli including the fibrogenic growth factor TGF-β. 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). TGF-β is usually activated 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). Activated TGF-β then binds with its receptors to exert its biological activities via activation of the TGF-β receptors, resulting in Smads phosphorylation (Derynck & Zhang, 2003). The phosphorylated Smads then bind to Smad4 to form a complex, which translocates into the nucleus to activate the transcription of many target genes, including CTGF (Chen et al., 2002; Akool et al., 2008) and TIMP-1(Akool et al., 2005 and 2008). Previously, we have published that CsA has the ability to induce the expression of the profibrotic genes CTGF and TIMP-1 by activating TGF-β/Smad signalling pathway in renal mesangial cells (Akool et al., 2008). The present work was designed to investigate a possible modulation of the TGF-β/Smad signaling pathway by CsA in rat liver.

MATERIALS AND METHODS

Animals

Rats (Male Wistar albino) weighing 200-240 g were housed in a 12h dark/light cycle animal facility with controlled humidity and constant temperature. The animals were fed a standard diet and water was supplied *ad libitum*. The animals were kept under observation for one week before the treatments for adaptation. The experimental protocol used in this study was approved by the Institutional Animal Ethics Committee.

Drugs and Chemicals

CsA was purchased from Sandoz Ltd, Basel, Switzerland. N-acetyl cysteine (NAC) was purchased from Sigma-Aldrich (USA). A neutralizing monoclonal $TGF\beta_{1-3}$ antibody (NAB), mouse IgG1 were purchased from R&D Systems (USA). Antibody specifically raised against phospho-Smad-2 was derived from Cell Signaling, USA. For immunohistochemistry, an antibody raised against phospho-Smad-2/3 was obtained from Santa Cruz Biotechnology, USA. Antibodies against histone deacetylase-1 (HDAC-1), anti-rabbit and anti-mouse HRPlinked IgGs were obtained from Santa Cruz Biotechnology, USA. The ECL system, and Hyperfilm were purchased from Amersham Pharmacia Biotech (USA). Rat transforming growth factor-$β_1$ (TGF-$β_1$), rat connective tissue growth factor (CTGF) and rat tissue

inhibitor of matrix metalloproteinase-1 (TIMP-1) ELISA kits were purchased from Kamiya Biomedical Company (USA), Cusabio (Wuhan, China), RayBiotech Inc. (Norcross, GA, USA) respectively.

EXPERIMENTAL DESIGN

Experiment 1:

The rats (6 animals in each group) received a single dose of CsA by intraperitoneal injection for different time points (1h, 4h, 8h and 24h) at a dose of 25mg/kg body weight. Control animals received the vehicle (castor oil) of CsA intraperitoneal. Blood samples were collected at the indicated time points for determination of plasma TGF-β levels. After terminal bleeding, animals were sacrificed by cervical dislocation. The liver was immediately dissected, washed with ice cold phosphate buffered saline (PBS) and kept at -20ºC for the analysis of Smads phosphorylation. Liver specimens were fixed in 10% neutral-buffered formal saline for immunohistochemical detection of p-Smad-2/3.

Experiment 2:

In experiments investigating the role of TGF-β and reactive oxygen species (ROS) in Smads phosphorylation induced by CsA, the animals were randomly divided into six groups, 6 animals in each. The first group (Control) was administered the vehicle of CsA intraperitoneal (i.p.). The second group received CsA (25mg/kg body weight i.p.). The third group was administered a neutralizing monoclonal TGFβ$_{1-3}$ antibody (0.5mg/kg body weight i.p.) one hour before CsA administration. The fourth group received control mouse IgG1 (0.5mg/kg body weight i.p.) one hour before CsA administration. The fifth group received NAC (40mg/kg body weight i.p.) one hour before CsA administration. The last group received NAC alone. Four hours after injection (based on the data from experiment 1), blood samples were collected for determination of plasma TGF-β levels. After terminal bleeding, animals were sacrificed by cervical dislocation. The liver was dissected immediately after death, washed with ice cold phosphate buffered saline (PBS) and kept at -20ºC for the analysis of phosphorylated Smad-2.

Experiment 3:

This experiment was designed to test first, the modulatory effect of CsA on CTGF and TIMP-1 expression. Second, the role of TGF-β and reactive oxygen species (ROS) in CTGF and TIMP-1 expression induced by CsA. The animals were treated with either vehicle (-), NAC, NAB or CsA alone or in combination with NAC, NAB or IgG1 as previously described in experiment 2. Twenty-four hours after injection, animals were sacrificed by cervical dislocation. The liver was immediately dissected, washed with ice cold phosphate buffered saline (PBS) and kept at -20ºC for the analysis of CTGF and TIMP-1 expression.

DETERMINATION OF PLASMA TGF-β LEVELS

The plasma level of TGF-β$_1$ was quantified by immunoassay kits (raised against rat TGFβ$_1$) according to the manufacturer's instructions (Kamiya Biomedical Company, USA).

Western blot analysis

Smad-2 phosphorylation and HDAC-1 were detected using Western blotting. For detection of the nuclear content of p-Smad-2, nuclear liver extracts containing 30-50 µg of protein were prepared and subjected to Western blotting analysis. Western blot analysis was performed as originally described by Laemmli (1970). Briefly, nuclear liver extracts (30-50µg) 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. For ensuring an equal loading of nuclear protein samples, the blots were reprobed with an anti-HDAC-1-specific antibody.

Immunohistochemical detection of p-Smad2/3 in rat liver

Paraffin-embedded sections of 4µm thickness were deparaffinised in xylene and rehydrated in graded ethanol solutions to distilled water. Sections were then incubated with 5% bovine serum albumin in Tris buffered saline for 2h for blocking of nonspecific immunoreactions. Sections were then incubated with the primary antibody p-Smad-2/3 (Santa Cruz Biotechnology, USA, Cat No. sc-11769-R) in a dilution of 1:125 at 4°C overnight for immunostaining. After washing the sections with TBS, they were incubated with goat anti-rabbit secondary antibody for 1 h at room temperature. Sections were then washed and incubated with diaminobenzidine (DAB) for 5 min at room temperature. The slides were counterstained with hematoxylin. Positive immunoreactions were visualized under a light microscopy. Negative control slides were included.

Determination of CTGF and TIMP-1 protein levels

The protein levels of CTGF and TIMP-1 in liver tissues were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Cusabio, Wuhan, China), (RayBiotech Inc., Norcross, GA, USA) respectively.

Statistical Analysis

Results are expressed as means $\pm$ 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 & discussion

To the best of our knowledge, this is the first study showing that administration of CsA causes a rapid activation of TGF-β/Smad signaling pathway in liver tissues. Furthermore, the present work demonstrates that Smads activation induced by CsA in liver tissues can be translated at the end to a clear up-regulation of Smad controlled gene expression of CTGF and TIMP-1. Previously, we have published that CsA has the ability to induce the expression of the profibrotic genes CTGF and TIMP-1 by activating TGF-β/Smad signalling pathway in renal mesangial cells (Akool et al., 2008). In the present work, a possible activation of Smad-2 by CsA was analyzed by monitoring the nuclear level of phosphorylated Smad-2 in liver tissues
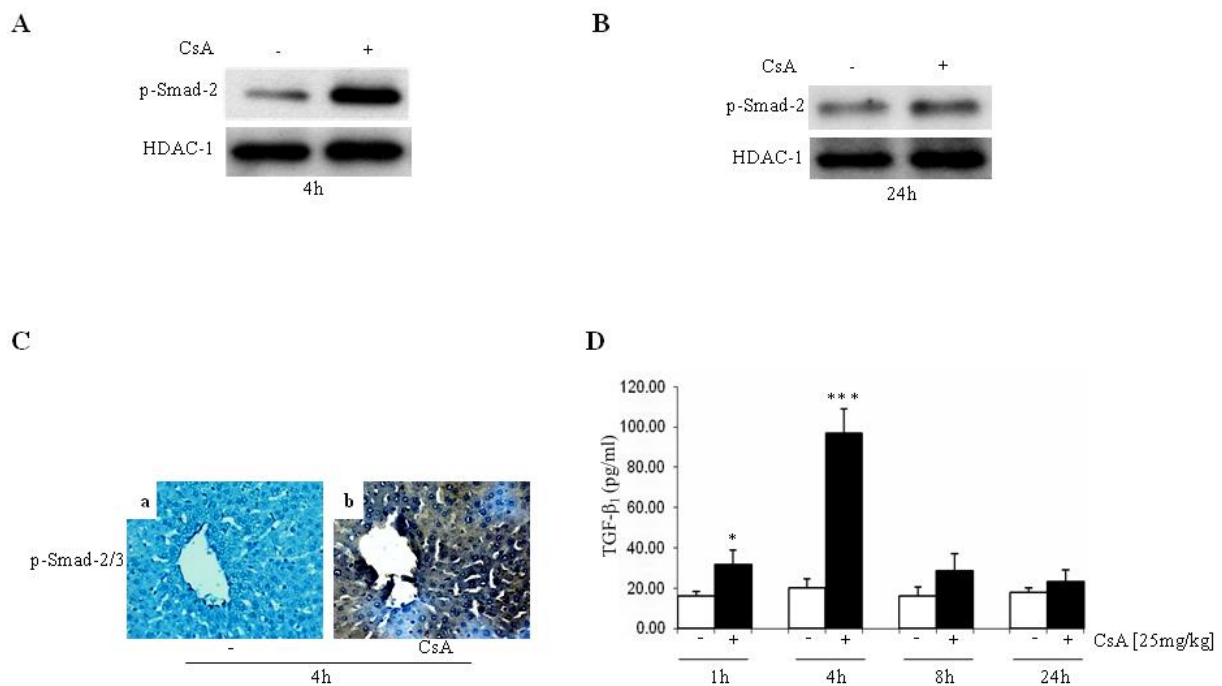

Fig. 1. CsA administration causes a rapid release of active TGF-β and Smads phosphorylation in liver tissues.
A, B. Smad-2 phosphorylation in liver tissues from rats treated with either vehicle (-) or CsA for the indicated time points. Nuclear liver extracts were subjected to Western blot analysis and probed with anti-phospho-Smad-2 and HDAC-1 antibodies. **C.** Immunohistochemical staining of p-Smad-2/3 in liver tissues from rats treated with either vehicle (-) or CsA for 4 h. a, Control group: shows normal architecture of liver with negative immunoreactivity of phosphorylated Smad2/3 (p-Smad2/3) [X400]. b, CsA group: shows p-Smad2/3 immunolocalized to centrolobular hepatocytes [X400]. **D.** Plasma levels of activated TGF-β_1 in rats treated with either vehicle (-) or CsA for the indicated time points. Data represent means ± S.D. (n=6), * $p < 0.05$, *** $p < 0.001$ versus control. The data shown are representative for six individually treated animals giving similar results.

as indicator for TGF-β/Smad signaling pathway activation. Time course experiments revealed that treatment of animals with CsA induced a rapid accumulation of phosphorylated Smad-2 within nuclear fractions after 4 h (Fig. 1A) which thereafter declined to a level of the control group after 24 h (Fig. 1B). Furthermore, Immunohistochemical staining of p-Smad-2/3 in liver tissues from rats treated with CsA shows p-Smad2/3 immunolocalized to centrolobular hepatocytes after 4 h (Fig. 1C). Most interestingly, measurement of TGF-β levels in plasma of CsA-treated animals revealed that the observed increase in the nuclear content of phosphorylated Smad-2 in liver tissues was associated with an increase in active TGF-β levels with a peak measured after 4 h, which thereafter declined to a level of vehicle treated animals (control) after 24h (Fig. 1D). To delineate the mechanism by which CsA activates Smad-2, the involvement of TGF-β in Smad-2 phosphorylation induced by CsA was tested using a neutralizing monoclonal TGF-β antibody. It was found that Smad-2 phosphorylation was highly reduced in those animals, which in addition to CsA, had been treated with
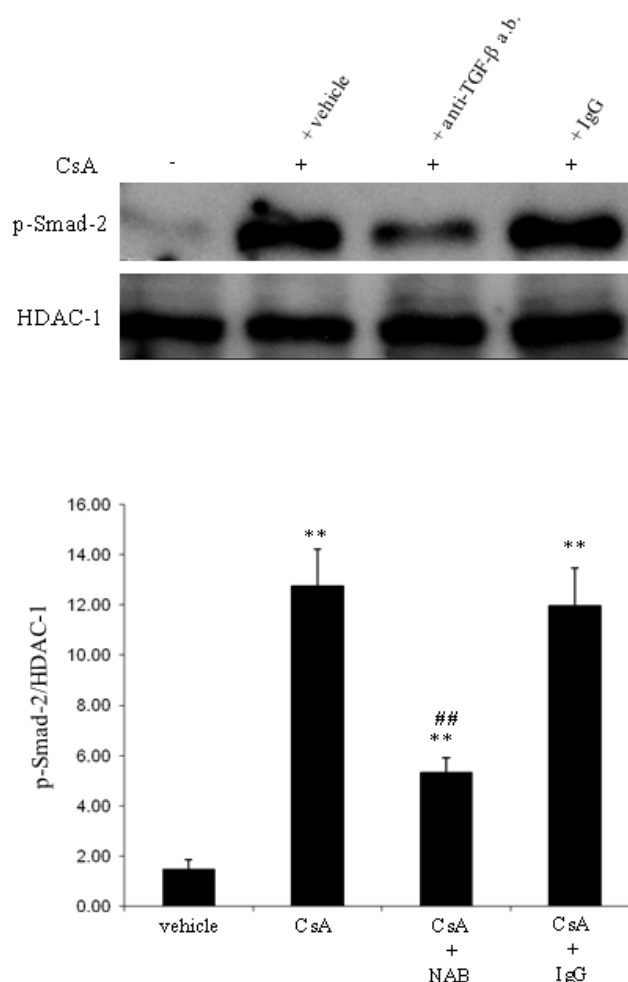

Fig. 2. CsA-triggered Smad-2 phosphorylation depends on TGF-β
Nuclear liver extracts from rats treated with either vehicle (-) or CsA alone or in combination with NAB or IgG1 (for 4 h) were subjected to Western blot analysis and probed with anti-p-Smad-2 and HDAC-1 antibodies. The

lower panel shows a densitometric analysis of nuclear p-Smad-2 relative to nuclear HDAC-1 level. Data represent means ± S.D. (n=6), ** $p < 0.01$ versus control, ## $p < 0.01$ versus CsA alone-treated animals.

a neutralizing TGF-β antibody (Fig. 2). However, administration of control IgG had no significant effect on Smad-2 phosphorylation induced by CsA (Fig. 2) indicating that TGF-β is involved in the rapid phosphorylation of Smad-2 by CsA. Physiologically, 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). Latent TGF-β is usually activated 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). Previously, it has been demonstrated that CsA generates ROS in different cell types (Krauskopf et al., 2002 and 2005; Akool et al., 2008). Furthermore, CsA has been shown to induce TGF-β activation and Smads phosphorylation via generation of ROS in renal cells (Akool et al., 2008). Therefore, the involvement of ROS in Smads phosphorylation induced by CsA in liver tissues was assessed.
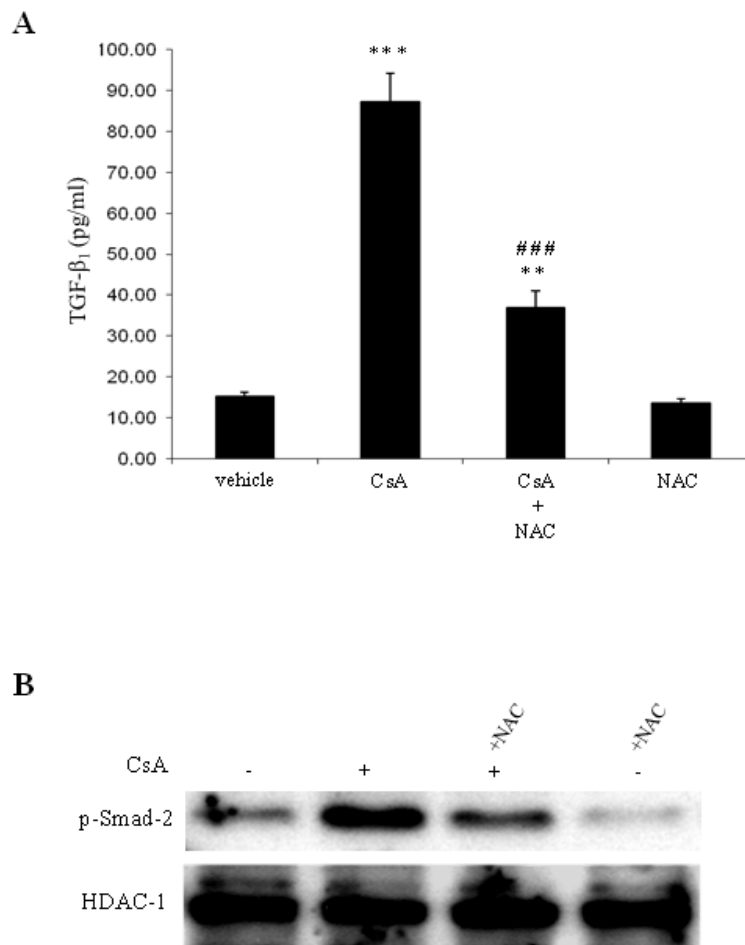
Fig. 3. TGF-β activation and Smad-2 phosphorylation induced by CsA is abrogated in the presence of NAC
A. Plasma levels of activated TGF-β_1 in rats treated with either vehicle (-) or NAC or CsA alone or in combination with NAC for 4 h. Data represent means ± S.D. (n=6), ** $p < 0.01$, *** $p < 0.001$ versus control, ### $p < 0.001$ versus CsA alone-treated animals. **B.** Nuclear liver extracts from rats treated with either vehicle (-) or NAC or CsA alone or in combination with NAC (for 4 h) were subjected to Western blot analysis and probed with anti-p-Smad-2 and HDAC-1 antibodies.

Interestingly, it was found that administration of the antioxidant NAC along with CsA significantly reduced TGF-β activation induced by CsA (Fig. 3A). As expected, the reduction in TGF-β release by NAC was accompanied with high reduction in Smad-2 phosphorylation induced by CsA (Fig. 3B) indicating that TGF-β activation and Smad-2 phosphorylation induced by CsA depends on ROS. To test whether the Smad-2 activation and translocation into the nucleus induced by CsA would functionally correlate with an up-regulation of Smad controlled gene expression, the protein levels of CTGF and TIMP-1 were determined by ELISA. As shown in Fig. 4, treatment of animals with CsA induces CTGF (Fig. 4A) and TIMP-1 (Fig. 4B). Most interestingly, concomitant administration of a neutralizing TGF-β antibody along with CsA caused a strong reduction in CsA-induced expression   of CTGF (Fig. 4A) and TIMP-1 (Fig. 4B). In contrast, administration of control IgG had no
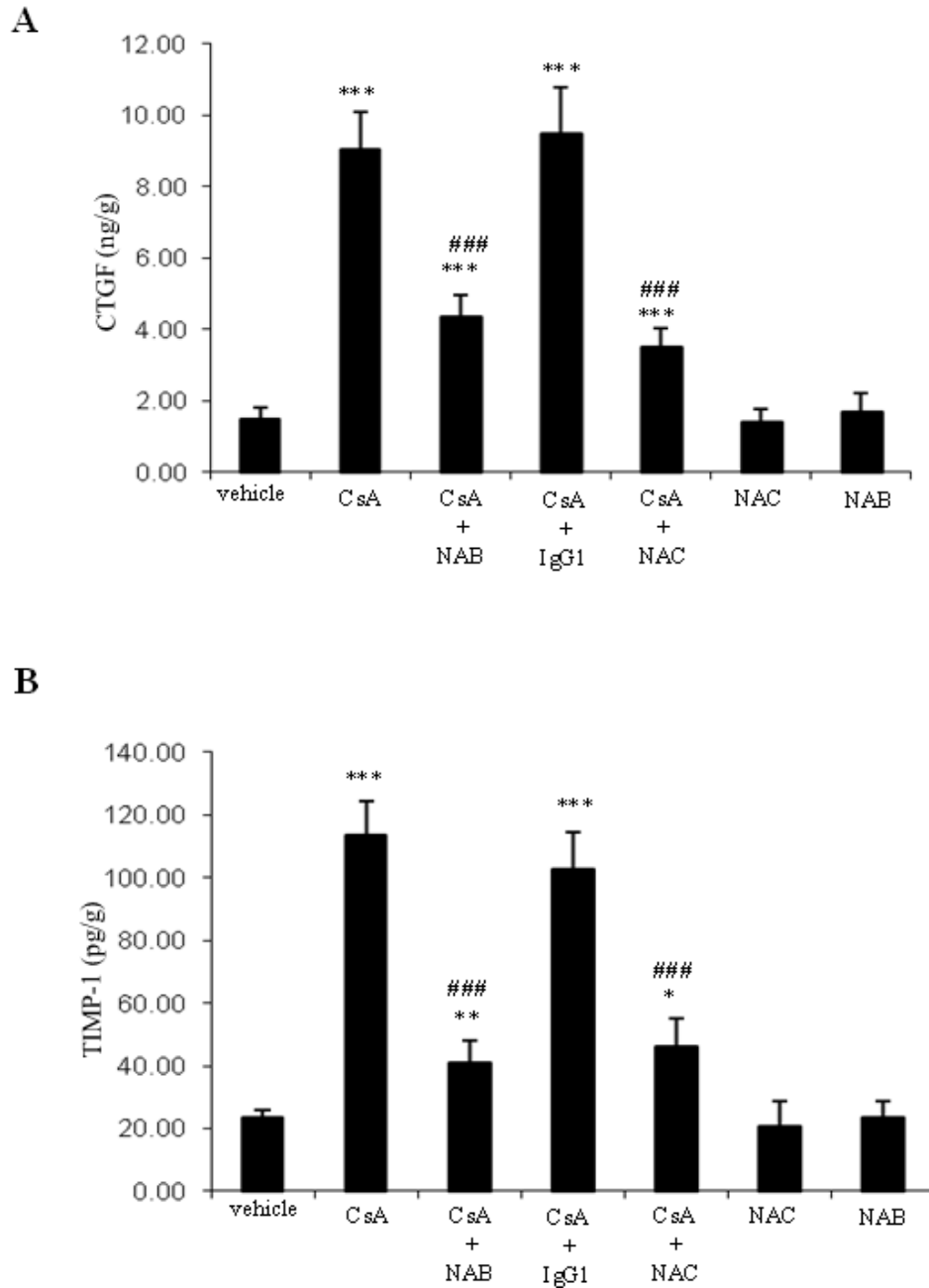
Fig. 4. CsA-induced CTGF and TIMP-1 expression is critically depends on TGF-β and ROS
Protein levels of CTGF (A) and TIMP-1(B) in liver tissues from rats treated with either vehicle (-), NAC, NAB or CsA alone or in combination with NAC, NAB or IgG1 for 24 h were determined by ELISA. Data represent means ± S.D. (n=6), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control, ### $p < 0.001$ versus CsA alone-treated animals.

significant effect on CsA-induced CTGF (Fig. 4A) and TIMP-1 (Fig. 4B) expression. Furthermore, concomitant administration of NAC along with CsA caused a strong reduction in CsA-induced CTGF (Fig. 4A) and TIMP-1 (Fig. 4B) expression. These data strongly suggest that Smad-2 activation by CsA is functionally relative and causative for an up-regulation of the profibrotic genes CTGF and TIMP-1 in TGF-β- and ROS-dependent

manner. In summary, administration of CsA causes a rapid activation of TGF-β/Smad signaling pathway in liver tissues via generation of ROS and subsequent activation of latent TGFβ that is sufficient to activate Smads which translocate into the nucleus and activates the transcription of the profibrotic genes CTGF and TIMP-1.

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السيكلوسبورين أ يحفز إشارات عامل النمو بيتا- سماد فى كبد الجرذان

للسيد الدكتور

دكتـــور/ السيد عاقول

مـــــــــن

قسم علم الأدويه والسموم- كلية الصيدلة- جامعة الأزهر

يعد السيكلوسبورين أ واحد من أهم مثبطات المناعة التى تستخدم بصورة كبيرة فى زراعة الاعضاء ولكن أثاره الجانبية العديدة تحد من استعماله اكلينيكيا. وقد وجد ان السيكلوسبورين أ يحفز إشارات عامل النمو- بيتا سماد (التى تلعب دوراً هاماً فى تليف الانسجة) فى خلايا الميزنجيال الكلوية.

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 كبد الجرذان من عدمه. كما استهدفت الدراسة ايضاً معرفة الميكانيكية الجزيئية لتحفيز هذه الاشارات بواسطة مثبط المناعة السيكلوسبورين أ.

وقد اتضح من خلال هذه الدراسة أن مثبط المناعة السيكلوسبورين أ ينشط اشارات عامل النمو بيتا- سماد فى كبد الجرذان, وذلك عن طريق انتاج فصائل الاكسجين الحرة , حيث انه قد لوحظ أن استخدام المواد المضادة للاكسدة تقلل الى حد كبير من نشاط عامل النمو بيتا 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.

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) . 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 كبد الجرذان والتى ينتج عنها زيادة فى انتاج عامل نمو الانسجة الضامة (CTGF) وكذلك مثبط الماتركس ميتالوبروتينيز-١(TIMP-1) اللذان يلعبان دوراً هاماً فى تليف الانسجة.