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THE MTOR INHIBITOR RAPAMYCIN INDUCES CTGF AND TIMP-1 EXPRESSION IN RAT KIDNEY: IMPLICATION OF TGF-β/SMAD SIGNALING CASCADE

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ABSTRACT

The mTOR-inhibitor rapamycin is a potent drug used as immunosuppressive agent. It is usually used either alone or in combination with calcineurin inhibitors. Despite its beneficial role in organ transplantation rapamycin in some cases can promote fibrosis in the kidney. In cultured mesangial cells, rapamycin has been shown to activate TGF-β/Smad signaling pathway in reactive oxygen species (ROS)-dependent manner. The present work was designed to test whether rapamycin-induced TGF-β/Smad signaling pathway and subsequent profibrotic gene expression observed in cultured mesangial cells would also occur in vivo. The present work demonstrates that treatment of animals with rapamycin causes an increase in the expression of the profibrotic genes connective tissue growth factor (CTGF) and tissue inhibitor of metalloproteinase-1 (TIMP-1) on mRNA and protein levels in dose-dependent manner. Furthermore, it was found that rapamycin has the ability to activate TGF-β/Smad signaling pathway in rat kidney as demonstrated by an increase in plasma TGF-β level and subsequent Smad phosphorylation. Moreover, it was found that administration of the antioxidant N-acetyl cysteine (NAC) along with rapamycin significantly reduced plasma TGF-β level and Smad-2 phosphorylation. Most interestingly, the expression of CTGF and TIMP-1 induced by rapamycin was highly reduced in the presence of either NAC or a neutralizing TGF-β antibody on mRNA and protein levels indicating that ROS and subsequent TGF-β activation are involved in CTGF and TIMP-1 expression induced by rapamycin. Finally, the present study demonstrates that rapamycin has the ability to induce CTGF and TIMP-1 expression in ROS and TGF-β-dependent manner in rat kidney (in vivo).

KEYWORDS: Rapamycin, CTGF, TIMP-1, TGF-β, rat kidney.

INTRODUCTION

Renal fibrosis has been found to be associated with excessive accumulation of extracellular matrix (ECM) due to insufficient matrix degradation.[1] ECM degradation is regulated by the action of two enzymes, the matrix metalloproteinases and the plasminogen activators and their intrinsic inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) and the plasminogen activator inhibitors, respectively.[2,3] TGF-β is a central player in the pathogenesis of renal fibrosis due to its ability to regulate the expression of these proteinase inhibitors.[4-6] In addition, TGF-β has been reported to induce the expression of several ECM components including connective tissue growth factor (CTGF). Furthermore, CTGF has been shown to stimulate ECM production.[7,8] TGF-β is usually secreted as latent complex (latent TGF-β) consisting of TGF-β covalently bound to latent TGF-β binding proteins (LTBP).[8,9] Activation of TGF-β is achieved by either proteolytic or nonproteolytic events [10] and in some cases may include a redox-sensitive mechanism.[11,12] In this respect, oxidation of the latency-associated peptide can cause a conformational change that releases TGF-β [11] Liberated or activated TGF-β then binds with its receptors to exert its biological activities via activation of the TGF-β receptors, resulting in Smad-2 and Smad-3 phosphorylation.[13] The phosphorylated Smad-2 and Smad-3 then bind to Smad4 to form a complex, which translocates into the nucleus and activates the transcription of many target genes, including CTGF[14,15] and TIMP-1.[15,16] Calcineurin inhibitors (CNI) are the most important immunosuppressive agents that are widely used in organ transplantation. However, the clinical use of CNI is strongly limited due to their nephrotoxicity which remains a major clinical problem. Rapamycin has both immunosuppressant and antiproliferative properties that has a unique mechanism of action distinct from that of CNI. Pharmacologically, rapamycin acts mainly through binding the immunophilin FK binding protein 12 (FKBP12). However, the rapamycin-FKBP12 complex has no effect on calcineurin phosphatase. Instead, it binds to the

mammalian target of rapamycin (mTOR), thereby inactivating the mTOR, a serine/threonine kinase which is critically involved in protein biosynthesis and cell cycle progression.[17] Cytokines activate mTOR, resulting in downstream events critical for cell cycle regulation. Rapamycin (sirolimus) blocks T-cell proliferation by blocking cytokine-mediated signal transduction pathways. Rapamycin has been established as an alternative immunosuppressant which is increasingly used to eliminate or at least lower CNI-induced adverse effects in the kidney.[18] However, despite this beneficial role rapamycin has been shown to induce TGF-β expression similar to CNIs.[19] Furthermore, it has been reported that rapamycin has a certain nephrotoxic potential especially when applied in combination with high doses of CNIs.[19,20] Moreover, it has been shown that rapamycin has the ability to induce the TGF-β/Smad signaling cascade and subsequent expression of profibrotic genes in cultured renal mesangial cells.[21] Thus, it was interesting to elucidate whether rapamycin would interfere with TGF-β/Smad signaling and thereby modulate the expression of the profibrotic genes in rat kidney (in vivo).

MATERIALS AND METHODS

Animals

Male Wistar albino rats weighing 170-220 g were housed in a 12 h dark/light cycle animal facility with controlled humidity and constant temperature. The animals were fed a standard diet pellets. 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

Rapamycin was purchased from Calbiochem (Schwalbach, Germany). N-acetyl cysteine (NAC) was purchased from Sigma-Aldrich (USA). Rat transforming growth factor-β (TGF-β, 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. A neutralizing monoclonal TGF-β_{1-3} antibody (NAB) and mouse IgG1 were purchased from R&D Systems (USA). Antibodies specifically raised against phospho-Smad-2 and total Smad-2 were derived from Cell Signaling, USA. Anti-rabbit HRPlinked IgGs was obtained from Santa Cruz Biotechnology, USA. The enhanced chemiluminescence (ECL) system was purchased from Amersham Pharmacia Biotech (USA).

Experimental Design

Experiment 1

To test the effect of rapamycin on CTGF and TIMP-1 expression, the rats (6 animals in each group) were treated with either vehicle (control) or rapamycin in different doses (1, 3, 6 mg/kg i.p.).[22] Twenty-four hours after injection, the animals were sacrificed by cervical dislocation. The kidney was dissected immediately after death, washed with ice cold PBS and kept at -20ºC for the analysis of CTGF and TIMP-1 expression.

Experiment 2

To investigate the effect of rapamycin on TGF-β activation and subsequent Smad phosphorylation, the rats (6 animals in each group) were administered a single dose of rapamycin (6mg/kg, i.p.) for different time points (1h, 2h, 4h, 6h, 8h and 24h). Control animals received the vehicle of rapamycin (i.p.). Blood samples were obtained at the indicated time points for determination of plasma TGF-β levels. After terminal bleeding, animals were sacrificed by cervical dislocation. The kidney was dissected immediately after death, washed with ice cold phosphate buffered saline (PBS) and kept at -20ºC for the analysis of Smad-2 phosphorylation.

Experiment 3

In experiments investigating the role of reactive oxygen species (ROS) and subsequent TGF-β activation in Smad phosphorylation induced by rapamycin, the animals were randomly divided into six groups, 6 animals in each. The first group (Control) was administered the vehicle of rapamycin (i.p.). The second group received rapamycin (6mg/kg, i.p.). The third group was administered a neutralizing monoclonal TGF-β_{1-3} antibody [0.5mg/kg body weight i.p.][15] one hour before rapamycin administration.The fourth group received control mouse IgG1 (0.5mg/kg body weight i.p.) one hour before rapamycin administration. The fifth group received NAC [40mg/kg body weight i.p.][23] one hour before rapamycin administration. The last group received NAC alone. Six hours after injection (based on the data from experiment 2), blood samples were collected for determination of plasma TGF-β levels. After terminal bleeding, animals were sacrificed by cervical dislocation. The kidney 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 4

This experiment was designed to investigate the involvement of ROS and subsequent TGF-β activation in CTGF and TIMP-1 expression induced by rapamycin. The rats were treated with either vehicle (control) or rapamycin or NAB or NAC or IgG1 or rapamycin in combination with either NAB or NAC or IgG1 as previously described in experiment 3. Twenty-four hours after injection, the animals were sacrificed by cervical dislocation. The kidney was dissected immediately after death, washed with ice cold PBS and kept at -20ºC for the analysis of CTGF and TIMP-1 expression.

Determination of plasma TGF-β level

The plasma level of TGFβ_1 was quantified by immunoassay kits (raised against rat TGFβ_1) according to the manufacturer's instructions (Kamiya Biomedical

Company, USA). The microtiter plate provided in this kit has been pre-coated with an antibody specific to TGF-β_1. Calibrators and samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for TGF-β_1. Next, Avidin conjugated to HRP was added to each microplate well and incubated. Then the 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added to each well. Only those wells that contain TGF-β_1, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulfuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm.

Western blot analysis

Phosphorylated Smad-2 and total Smad-2 were detected using Western blotting. Total kidney 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 described previously.[15]

Real-time PCR

mRNA transcripts of CTGF and TIMP-1 were detected using ABI prism 7700 Real-time PCR system (Applied Biosystems). Total RNA was extracted from kidney tissues using TRI-Reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. Total RNA (1µg) was reverse transcribed to cDNA using Moloney virus reverse transcriptase (Applied Biosystems). cDNA was amplified as follows: 2.5µl cDNA, 0.2µM of each primer, 12.5µl SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 25µl. The following primers were used:CTGF: 5′-CAGGCTGGAGAAGCAGAGTCGT-3′ (forward), 5′-CTGGTGCAGCCAGAAAGCTCAA-3′ (reverse); TIMP-1: 5′-ATAGTGCTGGCTGTGG GGTGTG-3′ (forward), 5′-TGATCGCTCTGGTAGCCCTTCTC-3′ (reverse); GAPDH: 5′-CCATTCTTCCACCTTTGATGCT-3′ (forward), 5′-TGTTGCTGTAGCCATATTCATTGT-3′ (reverse).[24] Real time PCR was done as follows: one initial step at 50 ºC for 2 min and 95 ºC for 2 min followed by 40 cycles at 95ºC for 15 seconds and 60ºC for 1 min. mRNA expression (as fold change from the GAPDH level) was determined by the $2^{-\Delta\Delta Ct}$ method.

Determination of CTGF and TIMP-1 protein levels

The protein levels of CTGF and TIMP-1 in kidney tissues were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Cusabio, Wuhan, China), (RayBiotech Inc., Norcross, GA, USA) respectively. This assay employs antibodies (specific for either rat CTGF or rat TIMP-1) coated on a 96-well plate. Standards and samples are added into the wells and CTGF or TIMP-1 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-rat CTGF or anti-rat TIMP-1 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of CTGF or TIMP-1 bound.

Statistical Analysis

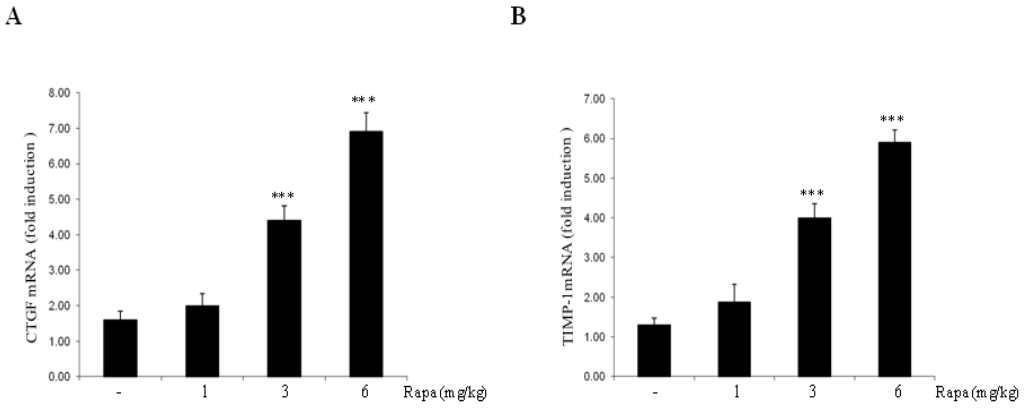
Results are expressed as means ± SD. One way ANOVA followed by Tukey-Kramer as a post-hoc test was used to analyze statistical significance among groups. *P*-values below 0.05 were considered as indication for statistically significant differences between conditions compared.

RESULTS AND DISCUSSION

In this report, we demonstrate that the immunosuppressive drug rapamycin potently induces the expression of CTGF and the intrinsic MMP-9 inhibitor TIMP-1 in rat kidney.Furthermore, it was found that rapamycin has the ability to induce the expression of the profibrotic genes CTGF and TIMP-1 by activating TGF-β/Smad signalling pathway. In the present work, a possible induction of CTGF and TIMP-1 by rapamycin was analyzed by monitoring the mRNA and protein levels of CTGF and TIMP-1 in kidney tissues. It was found that treatment of animals with rapamycin induced CTGF and TIMP-1 expression on mRNA (Fig. 1) and protein (Fig. 2) levels in dose-dependent manner. These results are in agreement with previous findings[21,25] in cultured mesangial cells indicating that rapamycin has the ability to induce the expression of CTGF and TIMP-1 not only in cultured mesangial cells but also in rat kidneys (in vivo). TGF-β and downstream Smad signaling pathways have been found to be the most important pathways involved in renal fibrosis via induction of the profibrotic genes CTGF and TIMP-1.[4-7] Previously, it has been shown that CTGF and TIMP-1 expression induced by rapamycin in cultured mesangial cells depends on Smad activation.[21,25] Furthermore, it has been demonstrated that rapamycin rapidly activates TGF-β/Smad signaling pathway in cultured mesangial cells via generation of reactive oxygen species (ROS).[21] Therefore, the possible modulatory effect of rapamycin on latent TGF-β activation in rats was first investigated. To this end, plasma levels of active TGF-β from rapamycin-treated animals were measured by ELISA. Measurement of TGF-β levels in plasma of rapamycin-treated animals revealed an increase in active TGF-β levels with a peak measured after 6 h, which thereafter declined to a level of vehicle treatedanimals after 24h (Fig. 3A). Most interestingly, rapamycin-induced TGF-β activation is accompained by Smad-2 phosphorylation after 6 h [peak level of TGF-β activation] (Fig. 3B) indicating that Smad-2 activation induced by rapamycin is critically depends on TGF-β activation. These results are in agreement with previous findings[21] in cultured mesangial cells indicating that rapamycin has the ability to rapidly activate TGF-β/Smad signaling pathway not only in cultured mesangial cells but also in rat kidneys

(in vivo). 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).[8,9] Latent TGF-β is usually activated by either proteolytic or nonproteolytic events[10] and in some cases may include a redox-sensitive mechanism.[11,12] In this respect, oxidation of the latency-associated peptide can cause a conformational change that releases TGF-β.[11] Previously, it has been demonstrated that rapamycin generates ROS in renal mesangial cells[21]Furthermore, rapamycin has been shown to induce TGF-β activation and Smad phosphorylation via generation of ROS in renal cells.[21] Therefore, the involvement of ROS in TGF-β activation and Smad Phosphorylation induced by rapamycin inrat kidney (in vivo) was assessed. Interestingly, it was found that administration of the antioxidant NAC along with rapamycin significantly reduced TGF-β activation induced by rapamycin (Fig. 4A) indicating that ROS is involved in TGF-β activation induced by rapamycin. As expected, the reduction in TGF-β release by NAC was accompanied with high reduction in Smad-2 phosphorylation induced by rapamycin (Fig. 4B) indicating that Smad-2 phosphorylation induced by rapamycin depends on ROS and subsequent TGF-β activation. Phosphorylated Smads with the co-Smad, Smad4, translocates into the nucleus. Subsequently, these complexes bind with the Smad binding elements (SBE), and thereby can activate the transcription of many TGF-β-induced target genes, including CTGF[14,15] and TIMP-1.[15,16] To test whether ROS formation and subsequent TGF-β/Smad signaling activation are involved in the expression of CTGF and TIMP-1, rats were treated with either vehicle (control) or rapamycin or NAB or NAC or IgG1 or rapamycin in combination with either NAB or NAC or IgG1. Interestingly, concomitant administration of a neutralizing TGF-β antibody along with rapamycin caused a strong reduction in rapamycin-induced expression of CTGF and TIMP-1 on mRNA (Fig. 5) and protein levels (Fig. 6). In contrast, administration of control IgG had no significant effect on rapamycin-induced CTGF and TIMP-1 expression (Fig. 5 & 6) indicating that TGF-β is involved in CTGF and TIMP-1 expression. Furthermore, concomitant administration of NAC along with rapamycin caused a strong reduction in rapamycin-induced CTGF and TIMP-1 expression on mRNA (Fig. 5) and protein levels (Fig. 6) indicating that ROS is involved in the expression of CTGF and TIMP-1. These data strongly suggest that Smad-2 activation induced by rapamycin is functionally relative and causative for an up-regulation of the profibrotic genes CTGF and TIMP-1 in ROS- and TGF-β-dependent manner. To the best of our knowledge this is the first time that rapamycin is shown to induce the expression of CTGF and TIMP-1 in rat kidney (in vivo).

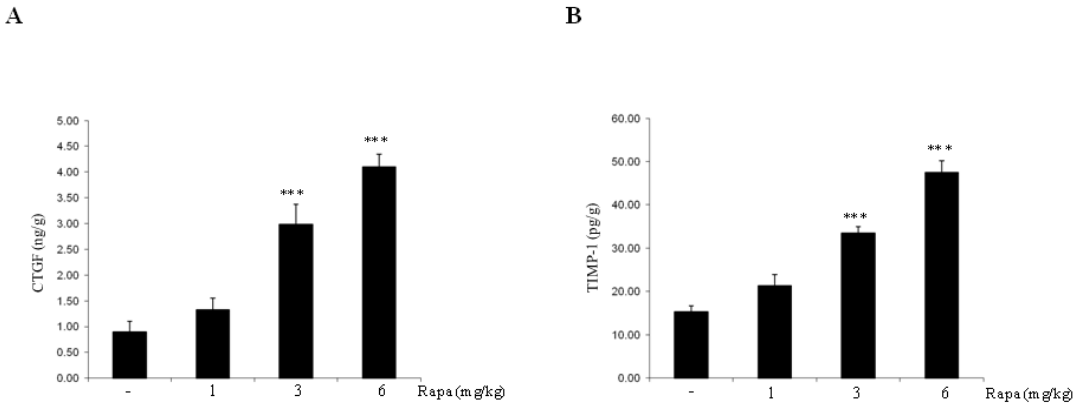
Figure 1



Rapamycin induces CTGF and TIMP-1 mRNA transcription in dose-dependent manner

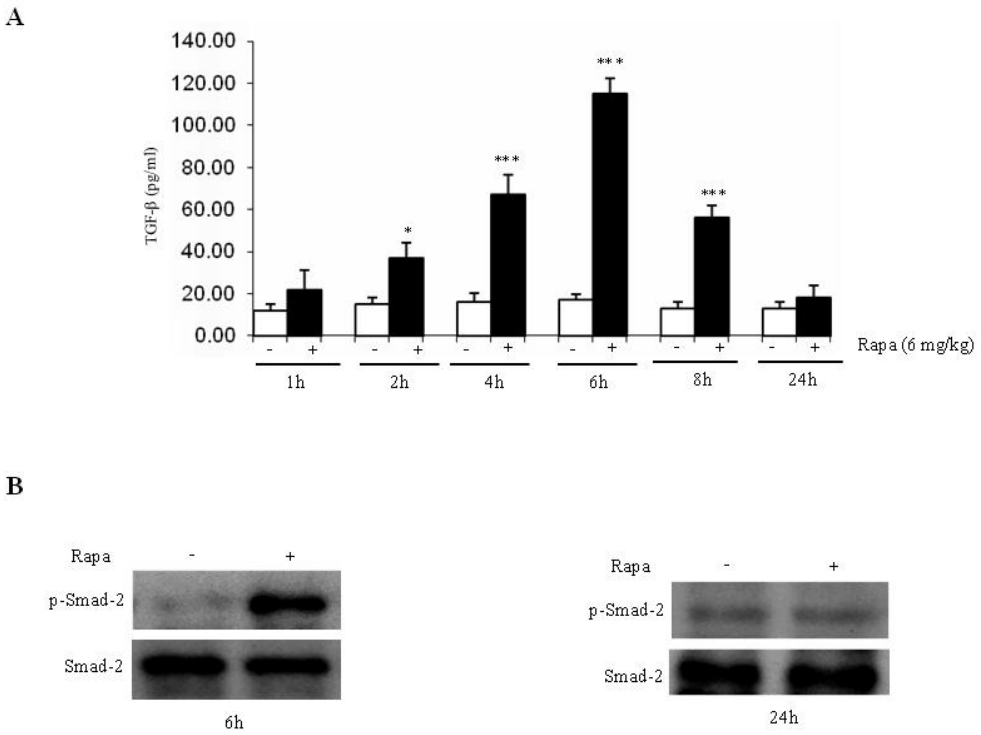
Total RNA was extracted from kidney tissues of rats treated with either vehicle (-) or different doses of rapamycin (Rapa) for 24h and mRNA expression of CTGF and TIMP-1 was determined by Real-time PCR analysis. CTGF and TIMP-1 mRNA was normalized to that of GAPDH and is shown as mean fold-induction. Data represent means ± S.D. (n=6), *** $p < 0.001$ versus control.

Figure 2



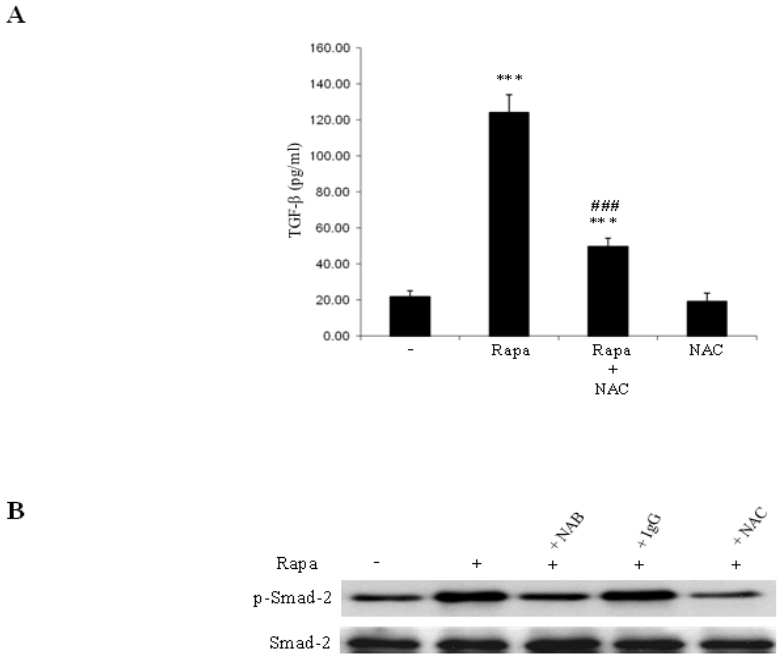
Rapamycin induces CTGF and TIMP-1 protein expression in dose-dependent manner
Protein levels of CTGF and TIMP-1 in kidney tissues from rats treated with either vehicle (-) or different doses of rapamycin (Rapa) for 24h were determined by ELISA. Data represent means ± S.D. (n=6), *** $p < 0.001$ versus control.

Figure 3



Time-dependent activation of TGF-β and Smad-2 by rapamycin.
A. Plasma levels of activated TGF-β in rats treated with either vehicle (-) or rapamycin (Rapa) for the indicated time points. Data represent means ± S.D. (n=6), * $p < 0.05$, *** $p < 0.001$ versus control. B. Smad-2 activation in kidney tissues from rats treated with either vehicle (-) or Rapa for the indicated time points. Total kidney extracts were subjected to Western blot analysis and probed with anti-phospho-Smad-2 and total Smad-2 antibodies.

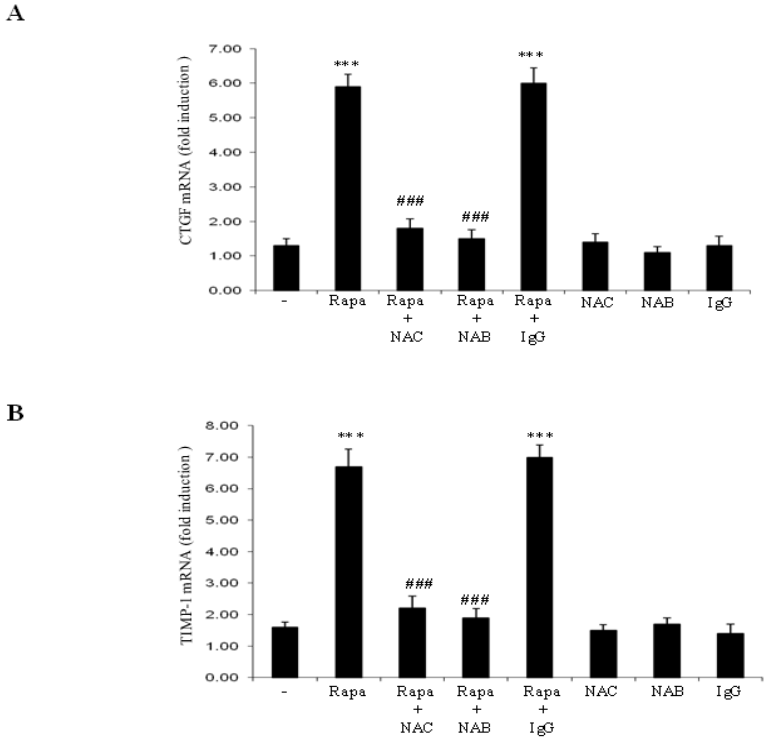
Figure 4



Rapamycin-induced TGF-β activation and Smad phosphorylation is abrogated in the presence of either NAC or NAB.

A. Plasma levels of activated TGF-β in rats treated with either vehicle (-) or NAC or rapamycin (Rapa) alone or in combination with NAC for 6 h. Data represent means ± S.D. (n=6), *** $p < 0.001$ versus control, ### $p < 0.001$ versus Rapa alone-treated animals. **B.** Total kidney extracts from rats treated with either vehicle(-) or Rapa alone or in combination with either a neutralizing $TGF\beta_{1-3}$ antibody (NAB) or IgG or NAC (for 6 h) were subjected to Western blot analysis and probed with anti-p-Smad-2 and total Smad-2 antibodies.

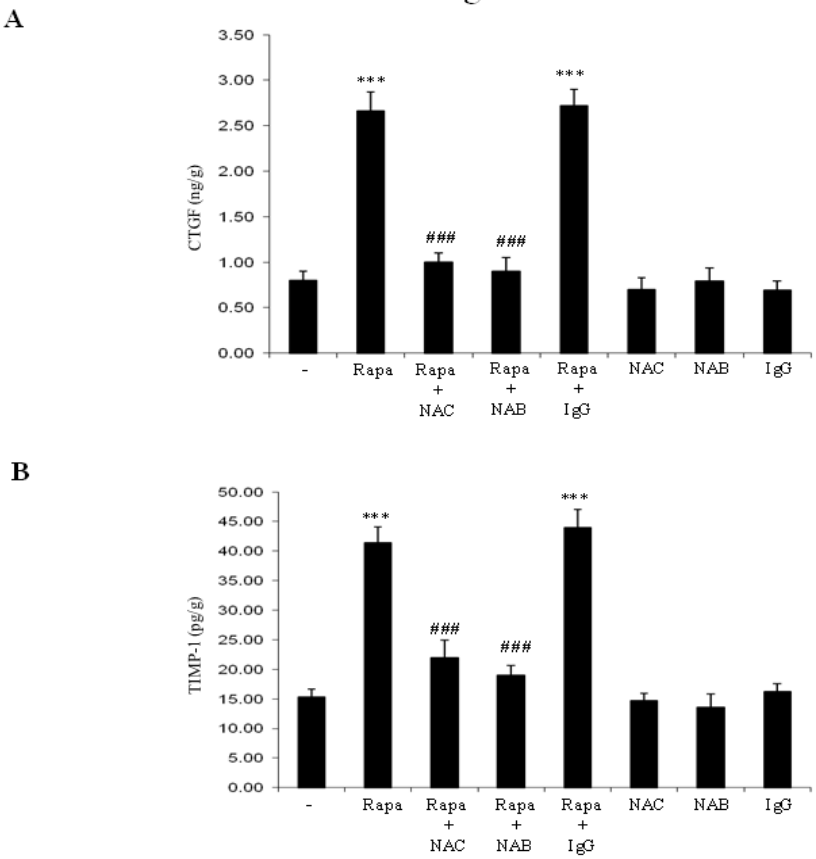
Figure 5



CTGF and TIMP-1 mRNA transcription induced by rapamycin is abrogated in the presence of either NAB or NAC.

Total RNA was extracted from kidney tissues of rats treated with either vehicle (-) or rapamycin (Rapa) or NAC or a neutralizing TGFβ$_{1-3}$ antibody (NAB) or IgG1 or Rapa in combination with either NAB or NAC or IgG1 for 24h and mRNA expression of CTGF (A) and TIMP-1 (B) was determined by Real-time PCR analysis. CTGF and TIMP-1 mRNA was normalized to that of GAPDH and is shown as mean fold-induction. Data represent means ± S.D. (n=6), *** $p < 0.001$ versus control, ### $p < 0.001$, versus Rapa alone-treated animals.

Figure 6



CTGF and TIMP-1 protein expression induced by rapamycin is abrogated in the presence of either NAB or NAC.

Protein levels of CTGF (A) and TIMP-1(B) in kidney tissues from rats treated with either vehicle (-) or rapamycin (Rapa) or NAC or a neutralizing TGFβ$_{1-3}$ antibody (NAB) or IgG1 or Rapa in combination with either NAB or NAC or IgG1 for 24 h were determined by ELISA. Data represent means ± S.D. (n=6), *** $p < 0.001$ versus control, ### $p < 0.001$ versus Rapa alone-treated animals.

CONCLUSION

This study shows that the mTOR inhibitor rapamycin induces CTGF and TIMP-1 expression in rat kidney (in vivo). Mechanistically, administration of rapamycin causes a rapid activation of TGF-β/Smad signaling pathway in ROS-dependent manner. Formation of ROS and subsequent activation of TGF-β/Smad signaling pathway are involved in the transcription of the profibrotic genes CTGF and TIMP-1.

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