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WHEAT GERM OIL AND VITAMIN E INHIBIT TGF-β/SMAD SIGNALING PATHWAY INDUCED BY TACROLIMUS (FK506) IN RAT KIDNEY

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ABSTRACT

Tacrolimus (FK506) is one of the most important immunosuppressive agents used in organ transplantation. However, its clinical use is strongly limited due to its nephrotoxicity. Transforming growth factor-β (TGF-β) and downstream Smad signaling have been found to play an important role in renal fibrosis via induction of profibrotic genes such as connective tissue growth factor (CTGF) and tissue inhibitors of matrix metallproteinases-1 (TIMP-1). FK506 has been shown to activate TGF-β/Smad signaling pathway in ROS dependent manner. The present work was designed to test the potential modulatory effect of wheat germ oil (WGO) as well as Vitamin E (Vit E) on TGF-β/Smad signaling pathway and subsequent CTGF and TIMP-1 expression induced by FK506 in rat kidney. It was found that FK506 administration causes a rapid activation of TGF-β/Smad signaling pathway as indicated by an increase in plasma TGFβ level and Smad-2 phosphorylation. In addition, activation of TGF-β/Smad signaling cascade is accompanied by an increase in Smad-dependent expression of CTGF and TIMP-1. Interestingly, concomitant administration of WGO as well as Vit E along with FK506 markedly inhibits TGF-β/Smad signaling pathway and subsequent CTGF and TIMP-1 expression that were associated with an increase in superoxide dismutase activity. Moreover, the immunosuppressive efficiency of FK506 neither affected by WGO nor Vit E.

KEYWORDS: **FK506; TGF-β/Smad signaling; Wheat Germ Oil**

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INTRODUCTION

Organ transplantation has become one of the most important fields in modern medicine. However, the risk of transplant rejection remains a major clinical problem. Transplant rejection can be prevented by the use of an immunosuppressive agent to suppress the reaction of the immune system to the transplanted organ. Calcineurin inhibitors are among the most efficient immunosuppressive drugs and therefore are widely used in transplantation and for the treatment of many inflammatory diseases including psoriasis, and rheumatoid arthritis. Tacrolimus (FK506) is one of the calcineurin inhibitors that suppress T-cell activation by inhibiting the cellular phosphatase calcineurin[1]. However, the clinical use of the calcineurin inhibitor FK506 is strongly limited by acute and chronic nephrotoxicity which is mainly characterized by glomerulosclerosis and tubulointerstitial fibrosis[2]. Renal fibrosis has been found to be associated with excessive accumulation of extracellular matrix (ECM) due to insufficient matrix degradation[3]. 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[4,5]. TGF-β is a central player in the pathogenesis of renal fibrosis due to its ability to regulate the expression of these proteinase inhibitors[6,7,8]. 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[9,10]. TGF-β is usually secreted as latent complex (latent TGF-β) consisting of TGF-β covalently bound to latent TGF-β binding proteins (LTBP)[10,11]. Oxidative stress has been shown to play an important role in TGF-β activation via oxidation of the latency-associated peptide, resulting in conformational change that releases TGF-β[12,13]. Liberated or activated TGF-β then binds with its receptor to exert its biological activity, resulting in Smad2/3 phosphorylation[14]. The phosphorylated Smad2/3 then bind to Smad4 to form a complex, which translocates into the nucleus and activates the transcription of many target genes, including CTGF[15,16] and TIMP-1[16,17]. Previously, it has been demonstrated that FK506 has the ability to rapidly activate TGF-β/Smad signaling pathway via generation of reactive oxygen species (ROS)[16]. The harmful effects of ROS induced by FK506 can be antagonized by using a powerful antioxidant agent[16,18]. Vitamin E (Vit E) has been found to be the most powerful antioxidant[19,20]. The richest known natural dietary source of Vit E is wheat germ oil (WGO)[21]. Furthermore, it has been reported that WGO intake results in a rapid increase in the content of Vit E in different rat tissues and gives high protection for these tissues against oxidative damage[22,23]. Therefore, the present study was designed to test the potential modulatory effect of WGO on FK506-induced TGF-β/Smad signaling pathway and subsequent CTGF and TIMP-1 expression in rat kidney. Vit E was used in the current study as a positive control antioxidant regimen.

MATERIALS AND METHODS

Animals

Male Wistar albino rats weighing 200-240 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 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

FK506 was purchased from Astellas Pharma Inc., Japan. WGO was purchased from SEDICO Pharmaceutical Co., 6 October City, Egypt. Vit E was purchased from Pharco Pharmaceuticals, Alexandria, Egypt. A neutralizing monoclonal TGFβ_{1-3} antibody (NAB) and mouse IgG1 were purchased from R&D Systems (USA). An antibody specifically raised against phospho-Smad-2 was derived from Cell Signaling, USA. An antibody raised against phospho-Smad-2/3 and anti-rabbit HRPlinked IgGs were obtained from Santa Cruz Biotechnology, USA. The enhanced chemi luminescence (ECL) system was 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. Superoxide dismutase (SOD) assay kit was purchased from Trevigen (USA). Interleukin-2 (IL-2) ELISA kit was purchased from Uscn Life Science Inc, Wuhan, China.

Experimental Design

Experiment 1

To investigate effect of FK506 on TGF-βactivation and Smad phosphorylation, rats (6 animals in each group) were administered a single dose of FK506 by intraperitoneal (i.p.) injection for different time points (1h, 2h, 4h, 8h and 24h) at a dose of 1mg/kg body weight[16]. Control animals received normal saline (i.p.). 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 kidneys were dissected immediately after death, washed with ice cold phosphate buffered saline (PBS) and kept at -20ºC for the analysis of Smad-2 phosphorylation.

Experiment 2

To investigate the role of TGF-β in Smad-2 phosphorylation induced by FK506 and to test the possible modulatory effect of WGO as well as Vit E on FK506-induced TGF-β activation and Smad phosphorylation, the animals were randomly divided into eight groups, 6 animals in each. The first group (Control) received normal saline (i.p.). The second group received FK506 (1mg/kg body weight i.p.). The third group received WGO (900mg/kg/day by oral gavage)[24] one day before and concurrently with FK506. The fourth group received Vit E (250mg/kg/day by oral

gavage)[25] one day before and concurrently with FK506. The fifth group was administered a neutralizing monoclonal TGFβ_{1-3} antibody (0.5mg/kg body weight i.p.)[16] one hour before FK506 administration. The sixth group received control mouse IgG1 (0.5mg/kg body weight i.p.) one hour before FK506 administration. The seventh group received WGO alone (as previously mentioned in group 3). The last group received Vit E alone (as previously mentioned in group 4). Four hours after injection (based on the data from experiment 1), blood samples were obtained for determination of plasma TGF-β levels. After terminal bleeding, animals were sacrificed by cervical dislocation. The left kidney was dissected immediately after death, washed with ice cold PBS and kept at -20ºC for the analysis of Smad-2 phosphorylation. The right kidney was fixed in 10% neutral-buffered formal saline for immunohistochemical detection of p-Smad-2/3.

Experiment 3

This experiment was designed to test first, the involvement of TGF-β in CTGF and TIMP-1 expression induced by FK506. Second, the possible modulatory effect of either WGO or Vit E on CTGF and TIMP-1 expression induced by FK506. Third, the potential modulatory effect of either WGO or Vit E on SOD activity. Fourth, the immunosuppressive efficiency of FK506 in the presence of either WGO or Vit E. The animals were treated with either FK506 or WGO or Vit E or a neutralizing monoclonal TGF-β_{1-3} antibody or IgG1 or FK506 in combination with either WGO or Vit E or a neutralizing monoclonal TGF-β_{1-3} antibody or IgG1 as previously described in experiment 2. Twenty-four hours after injection, blood samples were obtained for determination of serum IL-2 levels. After terminal bleeding, animals were sacrificed by cervical dislocation. The kidneys were dissected immediately after death, washed with ice cold PBS and kept at -20ºC for the analysis of CTGF and TIMP-1 expression as well as SOD activity.

Determination of plasma TGF-β level

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

Phosphorylated Smad-2 (p-Smad-2) and total Smad-2 in total kidney extracts were analysed using Western blot analysis as described previously[17]. Briefly, total kidney extract (30-50µg) was mixed with an equal volume of 2x electrophoresis sample buffer and incubated for 10 minutes at 95°C for denaturation. After separating the protein mixture by gel electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane by semi-dry electroblotting. After 1h blocking in 5% bovine serum albumin in Tris-buffered saline containing 0.05% Tween, the membrane was incubated with the primary antibody overnight at 4°C. Following incubation with secondary antibody (coupled to horseradish peroxidase), Signals were detected using the ECL system.

Immunohistochemical detection of p-Smad2/3 in rat kidneys

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.

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).[26] 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 95 ºC for 15 seconds and 60 ºC 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

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.

Determination of SOD Activity

SOD activity in renal tissue was determined by assay kit according to the manufacturer's instructions (Trevigen, USA). Briefly, superoxide radicals generated from the conversion of xanthine to uric acid and hydrogen peroxide by xanthine oxidase, converts nitroblue tetrazolium (NBT) to NBT-diformazan. NBT-diformazan absorbs light at 550 nm. SODs reduce superoxide radical concentrations and thereby lower the rate of NBT-diformazan formation. The extent of reduction in the appearance of NBT-diformazan is a measure of SOD activity.

Assessment of Serum level of IL-2

The level of IL-2 in serum was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Uscn Life Science Inc, Wuhan, China).

Statistical Analysis

Results are expressed as means ± SD. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer as a post-hoc test. *P*-values below 0.05 were considered as an indication for statistically significant differences between conditions compared.

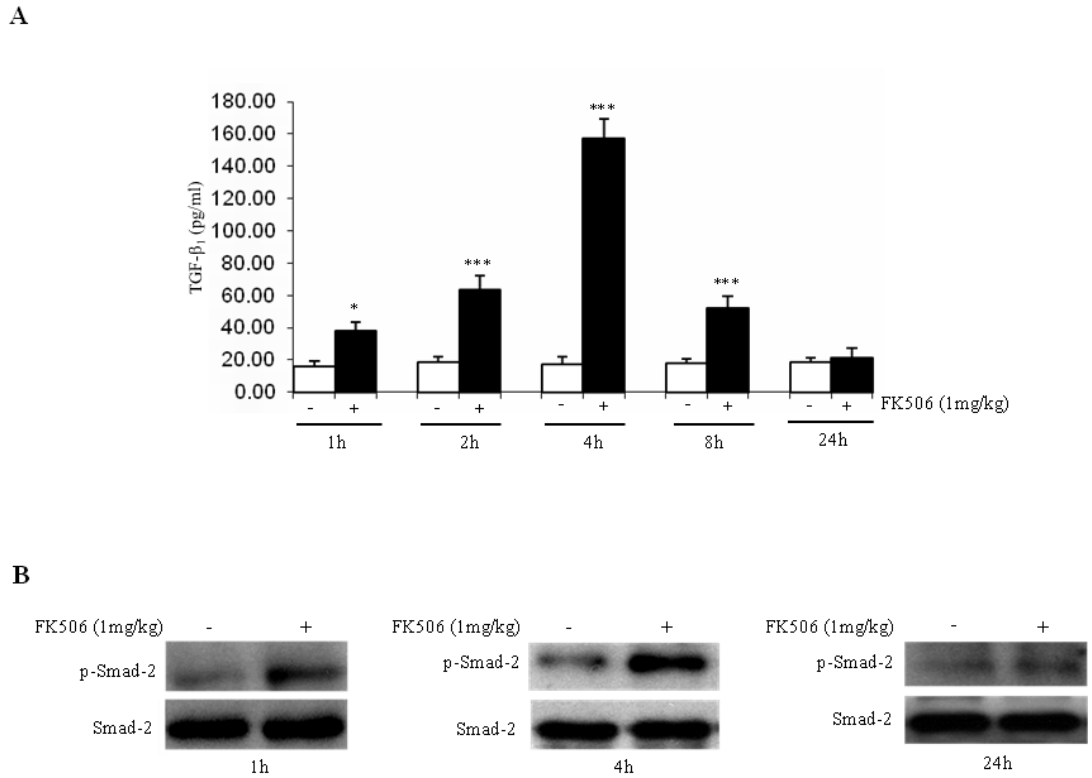
RESULTS

1. FK506 induces TGF-β activation and Smad-2 phosphorylation in rat kidney

Measurement of TGF-β levels in plasma of FK506-treated animals revealed an increase in active TGF-β levels with a peak measured after 4h, which thereafter declined to a level of vehicle treated animals after 24h (Fig 1A). Furthermore, TGF-β activation induced by FK506 is accompanied with an increase in Smad-2 phosphorylation after 4h (Fig 1B).

Figure 1

Time-dependent activation of TGF-β and Smad-2 by FK506



A. Plasma levels of activated TGF-β_1 in rats treated with either vehicle (-) or FK506 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 FK506 for the indicated time points. Total kidney extracts were subjected to Western blot analysis and probed with anti-phospho-Smad-2 antibody. For ascertainment that the total level of Smad-2 remained unchanged, blots were stripped and reprobed with anti-Smad-2. The data shown are representative for six individually treated animals giving similar results.

2. Smad2 phosphorylation induced by FK506 is TGF-β dependent

To check the involvement of TGF-β in Smad-2 phosphorylation induced by FK506, rats were pretreated with either a neutralizing monoclonal TGF-β_{1-3} antibody (NAB) or IgG1 before FK506 administration. As shown in Fig 2, Smad-2 phosphorylation induced by FK506 was highly reduced in the presence of NAB. However, administration of IgG1 had no significant effect on Smad-2 phosphorylation induced by FK506.

3. WGO as well as Vit E inhibits Smad-2 phosphorylation induced by FK506

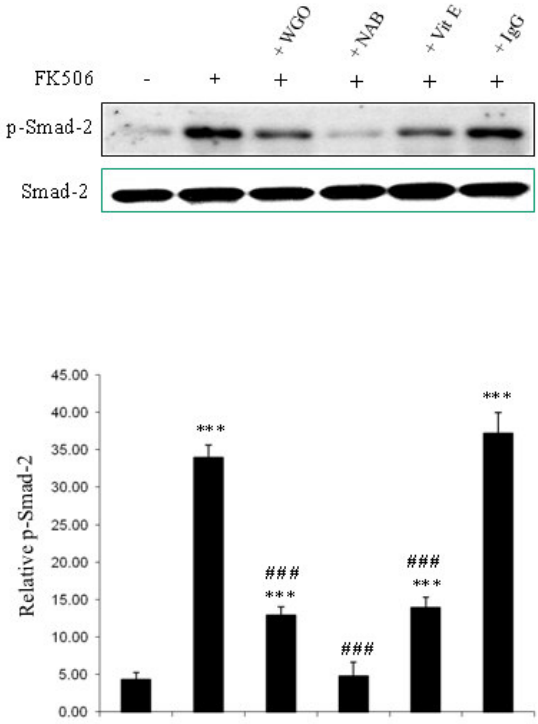
Activation of TGF-β is usually accompanied with an increase in Smad phosphorylation. Therefore, we checked whether Smad-2 phosphorylation induced by FK506 would also be modulated in the presence of either WGO or Vit E. As demonstrated in Fig 2, treatment of animals with FK506 induced a rapid phosphorylation of Smad-2. However, concomitant administration of either WGO or Vit E along with FK506 caused a strong reduction in Smad-2 phosphorylation

4. WGO as well as Vit E inhibits TGF-β activation and subsequent Smad-2/3phosphorylation induced by FK506 in rat kidney.

As shown in Figure 3A, treatment of rats with FK506 produced a marked activation of TGF-β. However concomitant administration of either WGO or Vit E along with FK506 significantly reduced TGF-β activation as compared with FK506-alone treated animals. No significant changes were observed in animals treated with either WGO or Vit E alone. Furthermore,

Figure 2

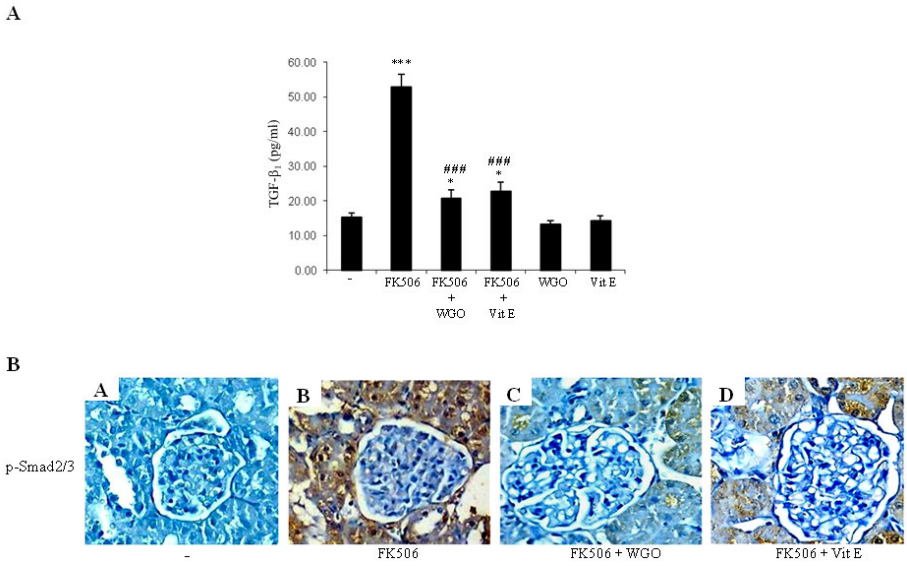
FK506-triggered Smad-2 phosphorylation is abrogated in the presence of either WGO or NAB or Vit E.



Total kidney extracts from rats treated with either FK506 or WGO or Vit E or a neutralizing TGFβ_{1-3} antibody (NAB) or IgG1 or FK506 in combination with either WGO or Vit E or NAB or IgG1 (for 4h) were subjected to Western blot analysis and probed with anti-p-Smad-2. For ascertainment that the total level of Smad-2 remained unchanged, blots were stripped and reprobed with anti-Smad-2. The lower panel shows a densitometric analysis of p-Smad-2 relative to the total Smad-2 level. Data represent means ± S.D. (n=6), *** p < 0.001 versus control, ### p < 0.001 versus FK506 alone-treated animals. The data shown are representative for six individually treated animals giving similar results

Figure 3

FK506-triggered TGF-β activation and Smad-2/3 phosphorylation is abrogated in the presence of either WGO or Vit E.



A. Plasma levels of activated TGF-β_1 in rats treated with either vehicle (-) or WGO or Vit E or FK506 alone or in combination with either WGO or Vit E for 4 h. Data represent means ± S.D. (n=6), * p < 0.05, *** p < 0.001 versus control, ### p < 0.001 versus FK506 alone-treated animals. B. Immunohistochemical staining of p-Smad-2/3 in kidney tissues from rats treated with either vehicle (-) or FK506 alone or in combination with either WGO or Vit E for 4h. a, Control group shows negative immunoreactivity with normal architecture of glomeruli and tubules (X400). b, FK506 group shows phosphorylated Smad2/3 (p-Smad2/3) immunolocalized to glomerular mesangial cells as well as proximal and distal convoluted tubules (X400). c, FK506+WGO group: p-Smad2/3 Immunostaining induced by FK506 was highly attenuated in the presence of WGO (X400). d. FK506+Vit E group: p-Smad2/3 immunostaining induced by FK506 was attenuated in the presence of Vit E (X400).

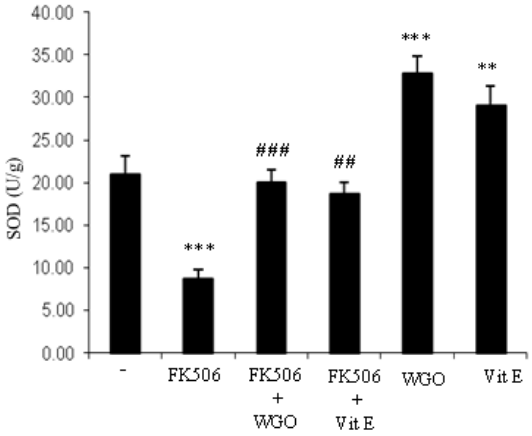
Smad-2/3 phosphorylation (p-Smad-2/3 immunostaining) induced by FK506 was highly reduced in animals pretreated with either WGO or Vit E (Fig 3B).

5. WGO as well as Vit E enhances SOD activity

To check the possible modulatory effect of either WGO or Vit E on SOD activity, animals were pretreated with either WGO or Vit E before administration of FK506. As shown in Fig 4, treatment of rats with FK506 produced a significant reduction in SOD activity.

Figure 4

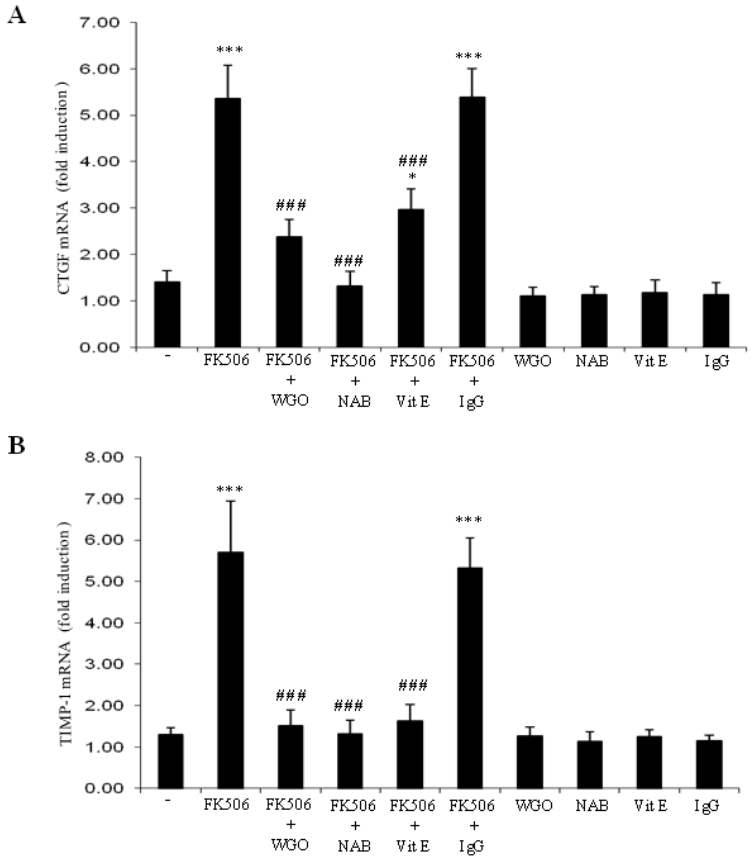
WGO as well as Vit E enhances renal enzymatic activity of SOD in male Wistar rats.



SOD activity in kidney tissues from rats treated with either vehicle (-) or WGO or Vit E or FK506 alone or FK506 in combination with either WGO or Vit E (for 24h) was determined by ELISA. Data represent means ± S.D. (n=6), ** $p < 0.01$, *** $p < 0.001$ versus control, ## $p < 0.01$, ### $p < 0.001$ versus FK506 alone-treated animals.

Figure 5

FK506-induced CTGF and TIMP-1 mRNA transcription is abrogated in the presence of either WGO or NAB or Vit E.



Total RNA was extracted from kidney tissues of rats treated with either FK506 or WGO or Vit E or a neutralizing TGFβ_{1-3} antibody (NAB) or IgG1 or FK506 in combination with either WGO or Vit E or NAB 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), * $p < 0.05$, *** $p < 0.001$ versus control, ### $p < 0.001$, versus FK506 alone-treated animals.

However, concomitant administration of either WGO or Vit E along with FK506 significantly increased SOD activity as compared to FK506 alone treated animals. Also, SOD activity is significantly increased in animals treated with either WGO or Vit E alone compared with the control group.

6. CTGF and TIMP-1 expression induced by FK506 is TGF-β dependent

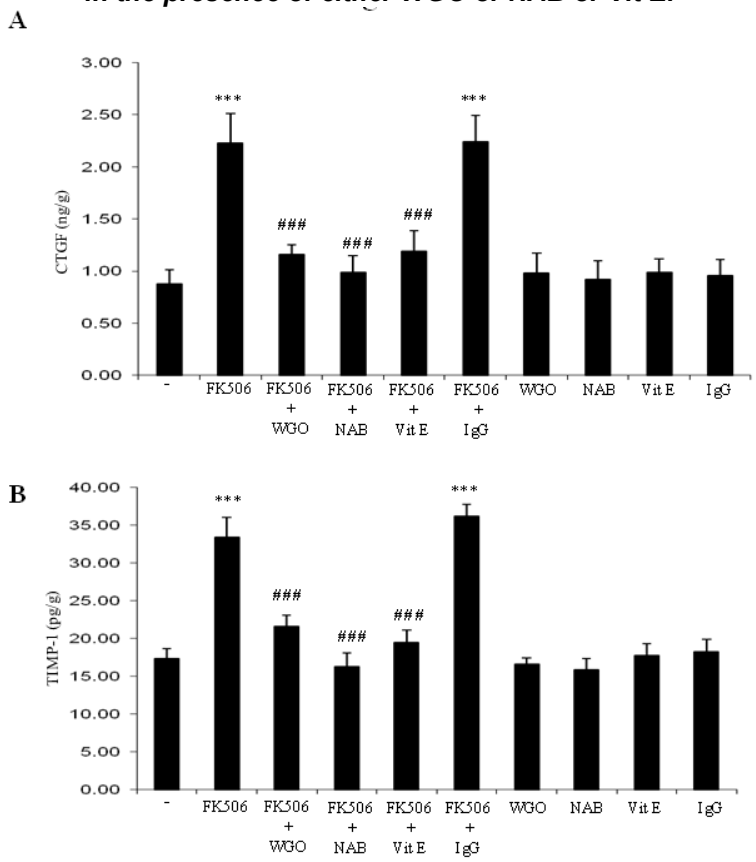
To test the involvement of TGF-β in CTGF and TIMP-1 expression induced by FK506, animals were pretreated with NAB or IgG1 before FK506 administration. As demonstrated in Fig 5 & 6, CTGF and TIMP-1 expression induced by FK506 was highly reduced in the presence of NAB on mRNA (Fig 5A & 5B) and protein (Fig 6A & 6B) levels. However, administration of IgG1 had no significant effect on CTGF & TIMP-1 expression induced by FK506 (Fig 5 & 6). No significant changes were observed in animals treated with either NAB or IgG1 alone (Fig 5 & 6).

7. WGO as well as Vit E inhibits CTGF and TIMP-1 expression induced by FK506

To test whether Smad2/3 activation induced by FK506 would functionally correlate with an up-regulation of Smad controlled genes expression of CTGF and TIMP-1, animals were pretreated with either WGO or Vit E before FK506 administration for 24h. AS shown in figure 5 & 6, treatment of rats with FK506 significantly induced CTGF and TIMP-1 expression on mRNA (Fig 5A & 5B) and protein (Fig 6A & 6B) levels. Most interestingly, CTGF and TIMP-1 expression induced by FK506 was highly reduced in the presence of either WGO or Vit E on mRNA (Fig 5A & 5B) and protein (Fig 6A & 6B) levels. No significant changes were observed in rats treated with either WGO or Vit E alone.

Figure 6

FK506-induced CTGF and TIMP-1 protein expression is abrogated in the presence of either WGO or NAB or Vit E.

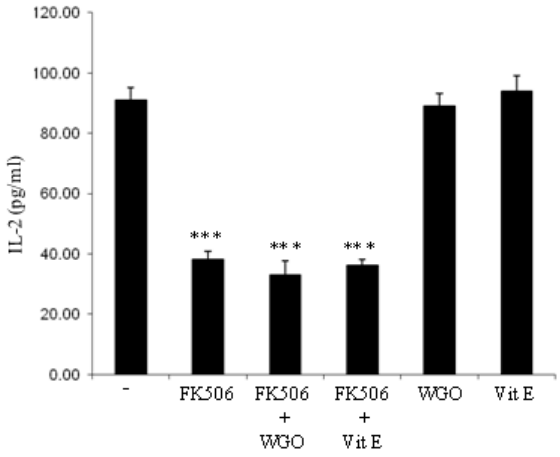


Protein levels of CTGF (A) and TIMP-1(B) in kidney tissues from rats treated with either FK506 or WGO or Vit E or a neutralizing TGFβ$_{1-3}$ antibody (NAB) or IgG1 or FK506 in combination with either WGO or Vit E or NAB or IgG1 for 24h were determined by ELISA. Data represent means ± S.D. (n=6), *** $p < 0.001$ versus control, ### $p < 0.001$ versus FK506 alone-treated animals.

8. WGO as well as Vit E has no effect on the immunosuppressive efficiency of FK506

Finally to test whether the immunosuppressive effect of FK506 would also be affected in the presence of either WGO or Vit E, serum level of IL-2 was evaluated. As demonstrated in Fig 7, treatment of rats with FK506 produced a significant reduction in serum IL-2 level. Interestingly, no significant changes were observed in rats treated with FK506 in combination with either WGO or Vit E as compared to FK506-alone treated animals.

Figure 7
The immunosuppressive efficiency of FK506 was not affected by either WGO or Vit E.



Serum levels of IL-2 in rats treated with either vehicle (-) or WGO or Vit E or FK506 alone or in combination with either WGO or Vit E for 24h were determined by ELISA. Data represent means ± S.D. (n=6), *** p < 0.001 versus control.

DISCUSSION

The calcineurin inhibitor FK506 is one of the most important immunosuppressive drugs. However its clinical use is accompanied by severe adverse effects including interstitial fibrosis and glomerulosclerosis[2]. TGF-β and downstream Smad signaling have been reported to play an important role in renal fibrosis via induction of the profibrotic genes CTGF and TIMP-1[6,9]. Oxidative stress has been shown to activate TGF-β by direct oxidative activation of latent TGF-β[12,13]. Previously, it has been demonstrated that FK506 via ROS generation activates TGF-β/Smad signaling pathway and thereby initiates Smad-driven gene expression of CTGF and TIMP-1[16]. The harmful effects of ROS induced by FK506 can be antagonized by using a powerful antioxidant agent[16,18]. WGO has been found to be the richest known natural dietary source of vitamin E[21]. Recently, it has been shown that WGO has the ability to inhibit liver toxicity induced by CsA via inhibition of ROS[25]. Therefore, it was interesting to check the possible modulatory effect of WGO on FK506-induced TGF-β/Smad signaling pathway and subsequent CTGF and TIMP-1 expression in rat kidney. Vit E was used in the current study as a positive control antioxidant regimen. In the present work, FK506 was found to activate TGF-β with maximal effect seen after 4h. Furthermore, TGF-β activation induced by FK506 is accompanied by phosphorylation of Smad-2 after 4h (peak level of TGF-β activation). These data are in agreement with previous finding[16]. To delineate the mechanism by which FK506 activates Smad-2, the involvement of TGF-β in Smad-2 phosphorylation induced by FK506 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 FK506, had been treated with a neutralizing TGF-β antibody, indicating that TGF-β is involved in the rapid phosphorylation of Smad-2 by FK506. In agreement with our finding, TGF-β has been shown to be the major factor involved in Smad-2 phosphorylation induced by FK506 in renal mesangial cells[16]. Latent TGF-β has been found to be sensitive to oxidative stress[13]. Furthermore, FK506 has been shown to induce TGF-β activation and Smad-2 phosphorylation via generation of ROS in renal cells[16]. Interestingly, the present work demonstrates that, WGO as well as Vit E has the ability to interfere with TGF-β activation and subsequent Smad-2/3 phosphorylation induced by FK506. Recently, it has been reported that mitochondrial cytochrome b-c_1 complex may represent the main sources of ROS (superoxide radical) induced by FK506 in the kidney[27]. To identify the mechanism by which WGO or Vit E inhibits TGF-β activation and subsequent Smad-2/3 phosphorylation induced by FK506, SOD activity was tested. Most interestingly, it was found that WGO as well as Vit E has the ability to induce SOD activity indicating that WGO may interfere with ROS-induced TGF-β activation and subsequent Smad phosphorylation via induction of SOD activity that scavenge superoxide radicals induced by FK506. In agreement with our finding, the cell permeant PEG-SOD has been shown to cause a marked attenuation in FK506-induced ERK phosphorylation in renal mesangial cells[27]. Physiologically, phosphorylated Smad-2/3 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[15,16] and TIMP-1[16,17]. In the present work, FK506 was found to induce CTGF and TIMP-1 expression on mRNA and protein levels indicating that Smad 2/3 activation induced by FK506 is functional relevant and causative for an up-regulation of the profibrotic genes CTGF and TIMP-1. In addition, CTGF and TIMP-1 expression induced by FK506 was found to be TGF-β dependent. Similar results were obtained in cultured mesangial cells[16]. Most interestingly, CTGF and TIMP-1 expression induced by FK506 were highly reduced in the presence of either WGO or Vit E on mRNA and protein levels indicating that WGO as well as Vit E has the ability to inhibit not only TGF-β/Smad signaling but also inhibits the expression of the profibrotic genes CTGF and TIMP-1

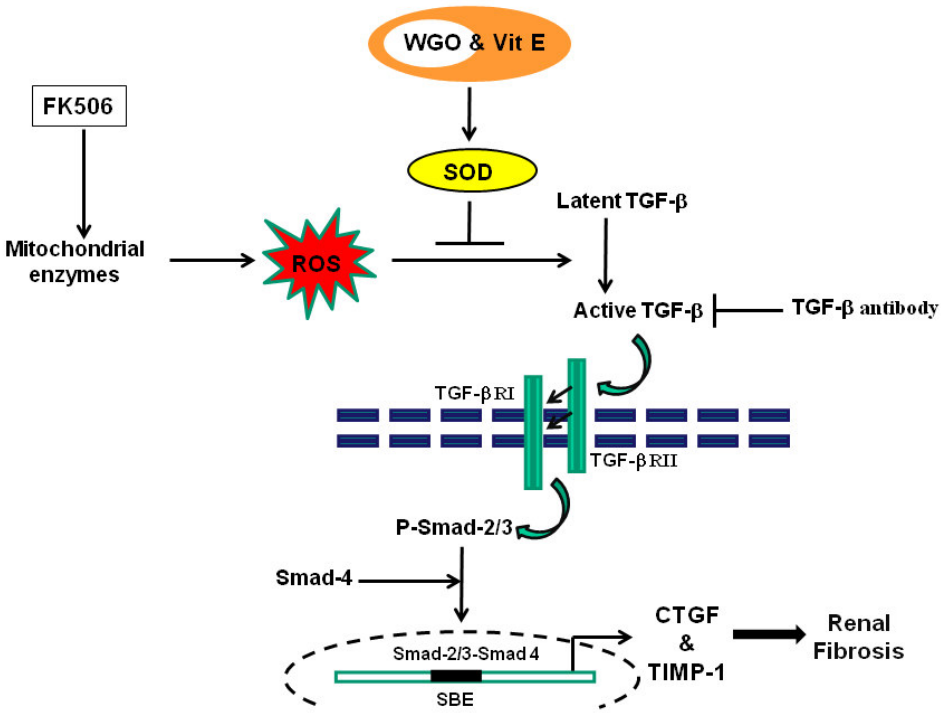
(the target genes of Smad 2/3 activation). Moreover, serum IL-2 level was evaluated as a marker of the immunosuppressive efficiency of FK506[28]. The present work demonstrates that, treatment of animals with FK506 produced a significant reduction in the serum level of IL-2 as expected. Interestingly, no significant changes were observed in animals treated with either FK506+WGO or FK506+Vit E as compared to FK506 alone treated animals indicating that the immunosuppressive effect of FK506 was not altered in the presence of either WGO or Vit E.

CONCLUSION

The present study demonstrates that WGO as well as Vit E has the ability to inhibit FK506-induced TGF-β/Smad signaling pathway and subsequent CTGF and TIMP-1 expression in rat kidney by increasing the renal enzymatic activity of SOD (Fig 8). These data may support the concept of using antioxidant therapy as a valuable approach for the prevention of FK506-induced renal fibrosis.

Figure 8

Schematic summary of the modulatory effect of either WGO or Vit E on TGF-β/Smad signling pathway and subsequent CTGF & TIMP-1 expresssion induced by FK506 in rat kidney.



FK506 generates ROS that causes TGF-β activation which in turn activates TGF-β receptors. Activation of the TGF-β receptors, results in Smad2 and Smad3 phosphorylation. The phosphorylated Smad2/3 then binds to Smad4 to form a complex, which translocates into the nucleus and activates the transcription of the profibrotic genes CTGF and TIMP-1. The diagram also demonstrates that WGO as well as Vit E via induction of SOD activity that scavenge ROS can inhibits TGF-β activation and subsequent Smad signaling, resulting in inhibition of CTGF and TIMP-1 expression.

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