Biochemical Pharmacology 83 (2012) 286–295

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Cyclosporin A and tacrolimus induce renal Erk1/2 pathway via ROS-induced and metalloproteinase-dependent EGF-receptor signaling

El-Sayed Akool [a], Stefan Gauer [b], Bashier Osman [a], Anke Doller [a], Sebastian Schulz [a], Helmut Geiger [b], Josef Pfeilschifter [a], Wolfgang Eberhardt [a,*]

[a] pharmazentrum frankfurt/ZAFES, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany
[b] Funktionsbereich Nephrologie, Zentrum der Inneren Medizin, Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Received 18 August 2011
Received in revised form 1 November 2011
Accepted 2 November 2011
Available online 9 November 2011

Keywords:
Calcineurin inhibitor
Heparin binding-epidermal growth factor
Metalloproteinases
Reactive oxygen species
Extracellular-regulated kinase

ABSTRACT

We previously demonstrated that the widely used immunosuppressive drugs cyclosporin A (CsA) and tacrolimus (FK506), independent of immunophilin binding, can activate profibrogenic transforming growth factor β (TGFβ)/Smad signaling cascades in rat renal mesangial cells (MC). Here we report that both peptidyl-prolyl *cis/trans* isomerase (PPIase) inhibitors activate the extracellular-signaling regulated kinase (ERK) a member of the mitogen activated protein kinase (MAPK) and induce a rapid and transient increase in ERK phosphorylation. The MEK inhibitor U0126, the reactive oxygen species (ROS) scavenger *N*-acetyl-cysteine (NAC), a cell-permeant superoxide dismutase (SOD) and stigmatellin, an inhibitor of mitochondrial cytochrome bc1 complex strongly attenuated the increase in ERK1/2 phosphorylation triggered by PPIase inhibitors. Moreover, neutralizing antibodies against heparin binding-epidermal growth factor (HB-EGF), and inhibition of the EGF receptor by either small interfering (si)RNA or AG1478, demonstrate that ERK activation by both PPIase inhibitors is mediated via HB-EGF-induced EGF receptor (EGFR) tyrosine kinase activation. The strong inhibitory effects achieved by GM6001 and TAPI-2 furthermore implicate the involvement of a desintegrin and metalloproteinase 17 (ADAM17). Concomitantly, the PPIase inhibitor-induced ADAM17 secretase activity was significantly reduced by SOD and stigmatellin thus suggesting that mitochondrial ROS play a primary role in PPIase inhibitor-induced and ADAM17-mediated HB-EGF shedding. Functionally, both immunosuppressants caused a strong increase in MC proliferation which was similarly impeded when cells were treated in the presence of NAC, TAPI-2 or AG1478, respectively. Our data suggest that CsA and FK506, via ROS-dependent and ADAM17-catalyzed HB-EGF shedding induce the mitogenic ERK1/2 signaling cascade in renal MC.

1. Introduction

The excessive synthesis and deposition of glomerular and interstitial extracellular matrix (ECM) is an important feature of many renal diseases characterized by glomerulosclerosis and interstitial fibrosis [1,2]. Functionally, the activated renal mesangial cells (MC) account as main producers of glomerular ECM which itself is indispensable for the structural and functional plasticity of an intact glomerulus [3,4]. In addition to many human kidney diseases, renal fibrosis is a major outcome of long-term immunosuppressive therapy with the peptidyl-prolyl *cis/trans* isomerase (PPIase) inhibitors cyclosporin A and tacrolimus (FK506) [5]. An increase in TGFβ plays a critical role in the PPIase inhibitor-induced nephropathy as has been convincingly demonstrated by the beneficial effects of TGFβ-neutralizing antibodies [6–10]. Mechanistically, both structurally dissimilar immunopressants impede T cell activation through a negative interference with the Ca^{2+}-induced activation of protein phosphatase 2B (calcineurin) thereby preventing dephosphorylation and activation of nuclear factor of activated T cells (NFAT) (for a review see [11]). With regard to adverse side effects of PPIase inhibitors, a potential involvement of reactive oxygen species (ROS) has been proposed by several studies ([12–14]; for review see [15,16]). In line with these studies, we previously demonstrated that CsA and FK506, independent of immunophilin binding, rapidly activate the canonical TGFβ-Smad signaling cascade by a ROS-triggered mechanism in rat MC [17]. Obviously, the direct source of PPIase inhibitor-induced ROS in rat MC is quite heterogeneous and involves the generation of either

Abbreviations: ADAM-17, a disintegrin and metalloproteinase 17; PPIase, peptidyl-prolyl *cis/trans* isomerase; CsA, cyclosporin A; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; MC, mesangial cells; NAC, *N*-acetyl cysteine; ROS, reactive oxygen species; siRNA, small interfering RNA.

* Corresponding author. Tel.: +49 69 6301 6954; fax: +49 69 6301 7942.
E-mail address: w.eberhardt@em.uni-frankfurt.de (W. Eberhardt).

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.11.001

superoxide and depends on the target investigated [17] or H_2O_2 [18]. In addition to the fibrogenic Smad pathway, TGFβ can induce extracellular signal-regulated kinases (ERKs) classically associated with growth factor-mediated signaling devices mainly implied in cell proliferation [19,20]. In addition, an elevation in ERK activity can enhance Smad activity in human MC thus suggesting that ERK activity is needed for an optimal response to TGFβ$_1$ [21]. However, mitogenic cell responses in most cases are initiated by the epidermal growth factor (EGF) or one of the EGF-like ligands including epiregulin, TGFα, amphiregulin, β-cellulin, neuregulins or heparin-binding EGF (HB-EGF). Each of these ligands displays overlapping but partially distinct binding affinities toward different ErbB receptors (for a review see: [22,23]). The EGF/ErbB family of receptor tyrosine kinases controls key cell functions including proliferation, migration, differentiation, and apoptosis (for review see [22–24]). Particularly, HB-EGF is one of the most prominent and best characterized mitogens in renal cells and of special interest since its expression is increased in experimental models and human forms of glomerulonephritides [25,26]. Mechanistically, transactivation of EGFR by HB-EGF shedding is target of many different external stimuli including activators of PKC and Raf-MAPK signaling cascades [26–29]. Conversely, a constitutive HB-EGF shedding was observed in tumor cells and therefore may constitute a target of novel anti-tumorigenic therapy [30]. Like other EGF-like ligands, HB-EGF is synthesized as inactive transmembrane precursor and the release of mature HB-EGF depends on the proteolytic cleavage by the action of metalloproteinases or a disintegrin and metalloproteinase (ADAM), namely ADAM17 [23,31–33]. However, the detailed mechanisms triggering the protease-dependent growth factor shedding are poorly understood. In addition to release of mature growth factor from the cell surface, the synthesis of HB-EGF is induced by phorbol esters, proinflammatory cytokines and hydrogen peroxide which are relevant for proliferation of MC in the course of glomerulonephritis [25].

Based on our previous findings that PPIase inhibitors via an activation of latent TGFβ initiate Smad-driven gene expression in renal MC [17], we aimed to elucidate whether both PPIase inhibitors would additionally activate mitogenic ERK signaling cascades. Interestingly, findings from experimental nephritis models indicate that indeed, a late accumulation of extracellular matrix (ECM) is preceded by MC proliferation and part of the physiological resolution phase in response to tissue injury [34,35]. Here we demonstrate for the first time, that a short-term treatment with PPIase inhibitors induces the mitogenic MAPK cascade via ROS-triggered EGF receptor signaling which inturn initiates MC proliferation.

2. Materials and methods

2.1. Materials

Human recombinant HB-EGF was purchased from Calbiochem (Schwalbach, Germany). A rabbit pan-specific HB-EGF antibody was purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). The PPIase inhibitors CsA and FK506 were from Axxora GmbH (Lörrach, Germany). AG1478, GM 6001, U0126, SP600125 and SB203580 and the TACE inhibitor TAPI-2 were obtained from Calbiochem (Schwalbach, Germany). Diphenylene iodonium (DPI), *N*-acetyl cysteine (NAC), catalase, polyethylene glycol-superoxide dismutase (PEG-SOD), and stigmatellin were obtained from Sigma Aldrich (Taufkirchen, Germany). Antibodies specifically raised against either phosphorylated and total ERK1/2, respectively and the antibodies against rat EGFR/HER-1 and phospho-EGFR (Tyr 1068) were from Cell Signaling (Frankfurt am Main, Germany). Antibodies against β-actin, anti-rabbit horseradish peroxidase-linked and anti-rabbit Alexa 488-linked secondary antibodies, as well as rabbit IgG were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). 5,-6-Dichloromethyl-2,7-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2-DCFDA), a cell-permeable indicator for ROS was from Molecular Probes (Karlsruhe, Germany).

2.2. Cell culture

Rat glomerular MC were grown as described [36]. All cell culture media and supplements were purchased from Life-Technologies (Karlsruhe, Germany). For experiments, serum-free preincubations were performed in Dulbecco's modified Eagle's medium supplemented with bovine serum albumin (BSA). In all experiments, the pharmacological inhibitors were applied half an hour prior to the addition of the PPIase inhibitors.

2.3. Western blot analysis

For detection of phosphorylated and total ERK1/2 levels, whole cell lysates were prepared as described previously [37]. Total cell extracts containing 30 μg of protein were prepared in SDS sample buffer, subjected to SDS-PAGE and Western blot analysis was performed by standard procedures. For detection of tyrosine-phosphorylated and total EGFR cells were directly lysed with SDS loading buffer without bromphenol blue and proteins subsequently transferred to polyvinylidene difluoride (PVDF) membranes before immunodetection.

2.4. RNA interference

Gene silencing of rat EGF receptor (GenBank accession no. NM_031507) was performed using customer siRNAs against rat EGF receptor from Qiagen (Hilden, Germany) and a control siRNA from Santa Cruz Biotechnology. Transfection of subconfluent MC was performed using the Oligofectamine reagent by following the manufacturer's instructions (Invitrogen, Karlsruhe, Germany).

2.5. Indirect immunofluorescence microscopy

Activation of mitogenic MAPK signaling was analyzed by measuring levels of phosphorylated ERK1/2 content by indirect immunofluorescence microscopy as described previously [38]. Images were analyzed using an inverse immunofluorescence microscope, BZ-7000 (Biozero, Keyence, Neu-Isenburg, Germany) equipped with a Zeiss Apo 20x/0.75 NA objective.

2.6. HB-EGF neutralization experiments

The impact of HB-EGF on the PPIase inhibitor-induced cell responses was tested by the use of a neutralizing HB-EGF antibody (R&D Systems). MC were pretreated with 20 μg of the neutralizing antibody for 60 min before stimulation with PPIase inhibitors. To exclude any unspecific inhibitory effects of the immunoglobulins, cells were treated with the same amount of rabbit IgG instead of the anti-HB-EGF antibody.

2.7. Detection of intracellular ROS production

Measurement of intracellular formation of ROS was done by using the cell permeant indicator CM-H_2DCFDA. For ROS measurement, serum-starved MC were stimulated for 1 h and after having removed the stimulation media, cells were washed once with Hank's balanced salt solution (HBSS). Trypsinized cells were

collected by a short centrifugation (1000 rpm), cell pellets resuspended in CM-H_2DCFDA (5 μM in HBSS) and incubated in the dark at 37 °C for 30 min. Subsequently, the cells were washed and returned to HBSS media and ROS generation was determined by measuring the formation of the oxidation product dichlorofluorescein (DCF) which was determined with a micro-plate fluorimeter (Wallac, Victor) by using excitation/emission wavelengths of 488 and 515 nm, respectively.

2.8. Measurement of ADAM17 (TACE) activity

ADAM17 (synonym TACE) activity was determined using the fluorimetric SensoLyte TACE (α-Secretase) activity assay kit (AnaSpec, San Jose, USA) following the manufacturer's specifications. This kit uses a FRET peptide substrate for the continuous measurement of ADAM17 enzyme activity. Upon cleavage of the FRET peptide by the active enzyme, the fluorescence of the intact FRET peptide 5-carboxyfluorescin which is normally quenched by QXL 520 is recovered and monitored at excitation/emission of 490/520 nm.

2.9. Measurement of cell proliferation

Proliferation of MC was determined by using a colorimetric 5′-bromo-2-deoxy-uridine (BrdU) labelling and detection kit III (Roche, Mannheim, Germany) following the manufacturer's instructions. Briefly, serum-starved MC were seeded on 6-well plates before treatment for 24 h with control medium or with different PPIase inhibitors in the presence or absence (vehicle) of different inhibitors. Subsequently, MC were labelled with BrdU for 6 h at 37 °C, and the incorporation of BrdU was determined by immunological detection of BrdU. Extinction of samples was measured at 492 nm using a microplate reader.

2.10. Statistical analysis

Results are expressed as means ± SD. Statistical analysis was performed using the Student's *t*-test and for multiple comparisons the ANOVA test for significance. The data are presented as relative induction compared with control conditions (*) or compared with CsA (#) or FK506 (§)-stimulated values. $P \leq 0.05$, ≤ 0.01, and ≤ 0.001 were considered significant

3. Results

3.1. CsA and FK506 activate ERK1/2 in glomerular MC

Previously, we demonstrated that the PPIase inhibitors CsA and FK506 independent of cyclophilin binding induce fibrogenic TGFβ-Smad signaling cascade by a ROS-dependent mechanism [17]. Moreover, we showed that the activation of Smad 2/3 by PPIase inhibitors partially depends on p38 MAPK activity which itself acts downstream of ROS. To test, whether PPIase inhibitors in addition to p38 MAPK, would exert any modulatory effects on the mitogenic MAPK signaling cascade, we monitored the phosphorylation state of ERK1/2 by using phospho-specific antibodies. According to results from our earlier reports, we chose standard concentrations of 1 μM CsA and 0.1 μM FK506, respectively which turned out to be most efficient without exerting significant cytotoxic effects on rat MC [17]. Time-course experiments revealed that stimulation of MC with CsA (1 μM) induced a rapid and significant increase in ERK1/2 phosphorylation already at 5 min and declined rapidly thereafter back to basal levels at 30 min (Fig. 1A). A similar induction profile was observed with FK506 (Fig. 1A). In contrast, the total ERK1/2 content did not change upon stimulation with either PPIase inhibitors (Fig. 1A). Similarly, an activation of ERK1/2 by CsA was observed by indirect immunofluorescence microscopy (Fig. 1B).

Activation of ERK1/2 by PPIase inhibitors is furthermore demonstrated by the strong inhibitory effects of U0126 (20 μM) an inhibitor of the upstream kinase MEK1/2 whereas the JNK inhibitor SP600125 (10 μM), exerted no inhibitory effect on CsA- or FK506-induced ERK1/2 phosphorylation (Fig. 1C). An additional inhibitory effect on drug-induced ERK phosphorylation was observed with the p38 inhibitor SB203580 (10 μM) thus indicating that activation of the p38 MAPK pathway is critically involved and upstream of ERK1/2 activation by PPIase inhibitors (Fig. 1C).

3.2. Activation of ERK1/2 by CsA is mediated via the EGFR

In a next step, we explored the possible involvement of the EGFR in PPIase inhibitor-induced ERK activation. For this purpose, expression of the EGFR (synonymously called erbB-1) was depleted by specific siRNA. MC transfected with an siRNA against the rat EGFR showed a strong reduction in EGFR expression when compared with cells transfected with a control siRNA (Fig. 2A). We found that depletion of EGFR expression by specific siRNA caused a clear augmentation in both, CsA- and FK506-triggered ERK phosphorylation (Fig. 2A). We furthermore tested the impact of CsA and FK506 on EGFR transactivation as determined by phosphorylation at tyrosine 1068 (EGFR-Tyr1068). CsA caused a clear and transient increase in EGFR phosphorylation at 5 min fully returned to basal levels at 20 min after the addition of CsA (Fig. 2B). In contrast, addition of recombinant EGF (50 pg/ml) which was used as a positive control caused a stronger and more sustained response when compared to CsA (Fig. 2B). By contrast, FK506 caused only a weak increase in EGFR-tyrosine phosphorylation the signal being hardly detectable by Western blot analysis (data not shown).

3.3. Activation of ERK1/2 by CsA and FK506 is prevented after neutralization of HB-EGF

The former data clearly indicate that the PPIase inhibitor-induced ERK1/2 phosphorylation depends on the transactivation of the EGFR. In a next step, we tested the impact of HB-EGF, which in MC accounts for a potent mitogenic agonist of the EGFR, by employing a neutralizing HB-EGF antibody [26,27]. Preincubation of MC with a neutralizing HB-EGF antibody (10 μg/ml) for 1 h followed by stimulation with CsA (1 μM) or FK506 (0.1 μM) caused an almost complete attenuation of PPIase inhibitor-induced ERK1/2 phosphorylation but interestingly, did not affect constitutive ERK1/2 phosphorylation in MC (Fig. 3A). In contrast, the addition of isotype-specific control IgG had no effect on either basal or on PPIase inhibitor-induced phospho-ERK1/2 levels (Fig. 3A). By contrast, pharmacological inhibition of EGFR tyrosine kinase activity by AG1478 (250 nM) caused an almost complete reduction of basal and stimulated ERK phosphorylation (Fig. 3A). These data indicate that CsA and FK506 can induce a canonical induction of EGFR-ERK1/2 signaling pathway which is specifically triggered by HB-EGF. Due to the fact that we could not detect HB-EGF in the conditioned media of PPIase inhibitor-treated MC, we compared the phospho ERK1/2 contents evoked by different amounts of recombinant HB-EGF with those which were induced upon stimulation with PPIase inhibitors. As shown in Fig. 3B, the increase in ERK phosphorylation induced by CsA or FK506 is weaker than that evoked by 10 pg/ml HB-EGF, a concentration which is at the lower detection limit of HB-EGF-specific ELISAs.

3.4. Activation of ERK1/2 by PPIase inhibitors depends on ROS

Based on our previous finding that PPIase inhibitors induce the Smad signaling cascade through an increase in ROS production

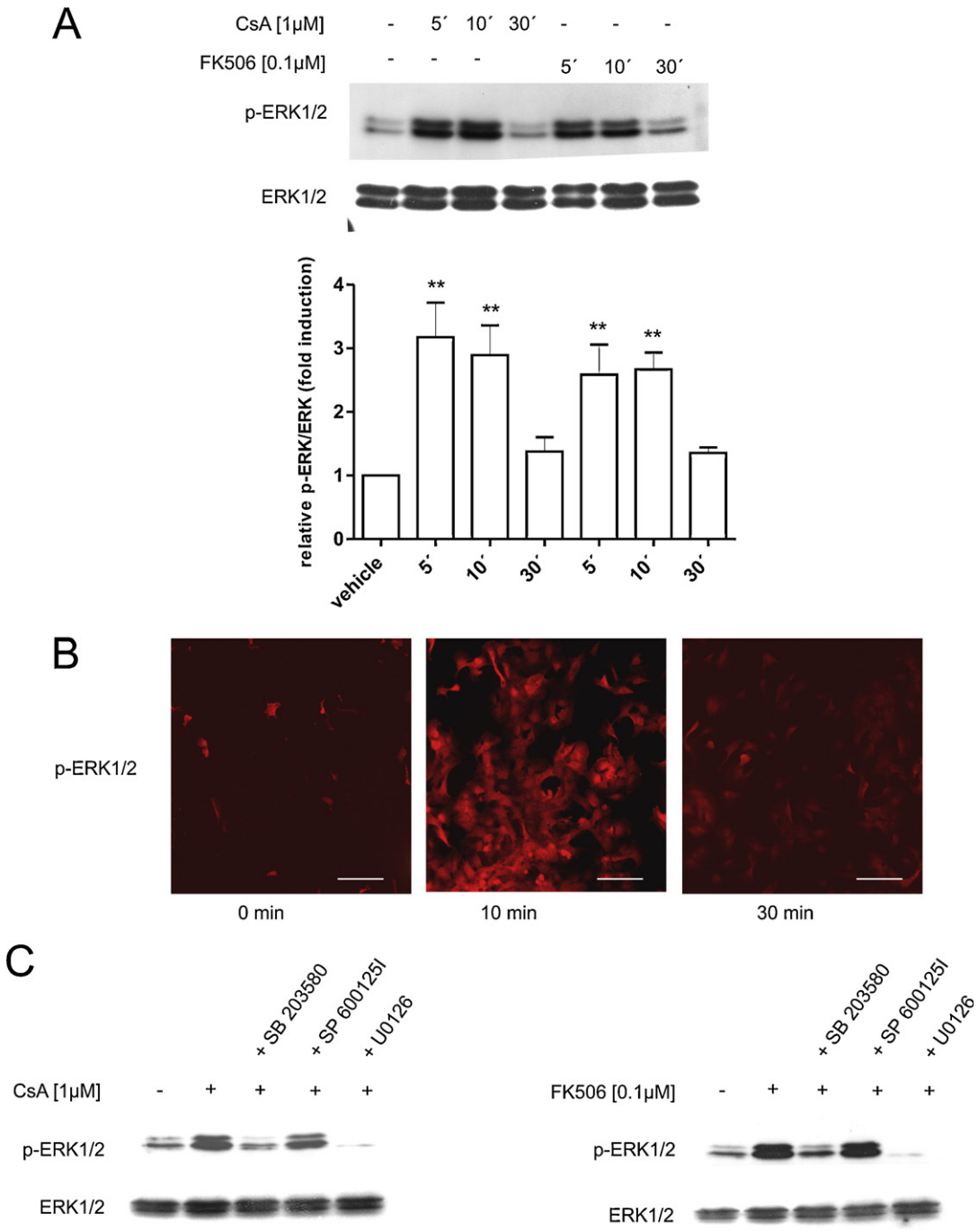

Fig. 1. CsA and FK506 cause a rapid and transient activation of ERK1/2 in renal MC. (A) Serum-deprived MC were stimulated with either vehicle (−) or with CsA (1 μM) or FK506 (0.1 μM) for the indicated time periods prior to cell lysis. Total protein (30 μg) was subjected to Western blot analysis and successively immunoblotted against phosphorylated (p-ERK1/2) and total ERK1/2 (ERK1/2). Data in the lower panel of (A) show a densitometric analysis of phospho-ERK1/2 relative to total ERK1/2 levels and represent means ± S.D. ($n = 3$). $^{**}p \leq 0.01$ versus unstimulated control. (B) Indirect immunofluorescence visualizing time-dependent increase in ERK1/2 phosphorylation by CsA. Quiescent MC were stimulated for 10 or 30 min before cells were fixed and stained with a phospho-specific ERK1/2 antibody and the secondary anti-mouse Cyc 3 antibody. Bar, 50 μm. (C) Effects of different MAPK inhibitors on CsA-induced ERK1/2 phosphorylation. Quiescent MC were pretreated for 30 min with vehicle (−) or with the indicated MAPK inhibitors before cells were stimulated for 10 min with (+) or without (−) 1 μM of CsA. The different MAPK inhibitors were applied at the following concentrations: SB203580 (10 μM), SP600125 (10 μM), and U0126 (20 μM). Western blot analysis was performed as described in (A). Data shown in (B) and (C) are representative of two independent experiments giving similar results.

[17], we wondered if CNI-induced ERK activation similarly would depend on ROS. For this purpose, we tested for modulatory effects by the ROS scavenger NAC (5 mM), or by diphenylene iodonium (DPI, 10 μM), an inhibitor of NADPH oxidases when both inhibitors were applied 30 min before the PPIase inhibitors. As shown in Fig. 4A, NAC caused an almost complete inhibition of PPIase inhibitor-induced ERK1/2 phosphorylation whereas DPI had no significant effect on PPIase inhibitor-triggered ERK1/2 activity.

To corroborate that NAC effects on ERK phosphorylation are due to changes in ROS generation, we measured cellular radical formation by using dichlorodihydrofluorescein (DCF), a cell-permeant ROS acceptor. Consistent with the data from Western blot analysis, both PPIase inhibitor induced a significant increase in ROS which was almost completely abrogated in the presence of NAC, but again not affected by DPI (Table 1). To identify the source of PPIase inhibitor-induced ROS formation, we employed further

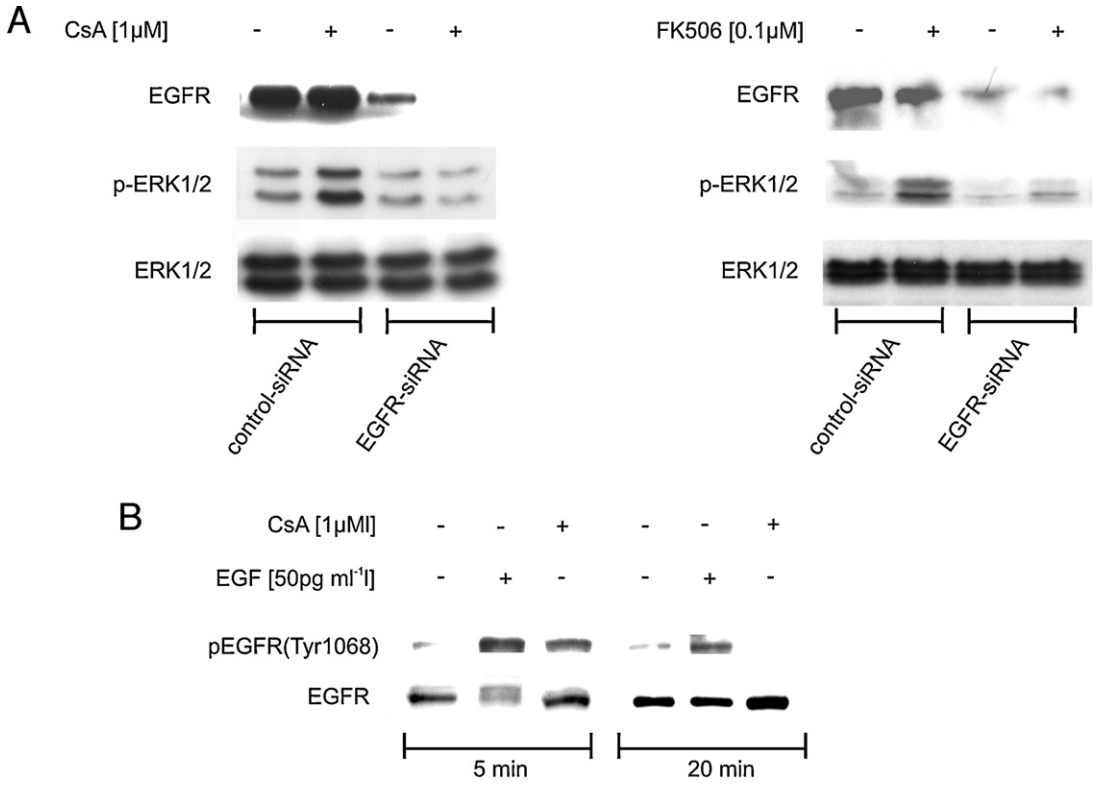

Fig. 2. CsA and FK506 induce ERK1/2 phosphorylation through a transactivation of the EGFR. (A) Serum-deprived MC were transfected with either a control siRNA (control-siRNA) or with siRNA duplexes specific for rat EGFR (EGFR-siRNA). 24 h after transfection MC were serum-starved for 16 h before being stimulated with either vehicle (–), or CsA (left panel) or FK506 (right panel) for 10 min as indicated. Subsequently, cells were lyzed for Western blot analysis and 50 μg of total cell lysates successively probed with anti-EGFR (EGFR), anti-phospho-ERK1/2 (p-ERK1/2) and anti-ERK1/2 (ERK1/2)-specific antibodies, respectively. Data shown are representative of two independent experiments giving similar results. (B) Epidermal growth factor receptor (EGFR) transactivation by EGF, CsA or FK506 as assessed by EGFR phosphorylation at Tyr-1068. Serum-deprived MC were treated for the indicated time points with vehicle (–) without or with either EGF (50 pg/ml) or with CsA (1 μM) before cells were lysed for Western blot analysis as described in Section 2. Western blots were successively probed with anti-phospho-EGFR (Tyr1068)-specific and anti-EGFR (ErbB-1)-specific antibodies. Data are representative for three independent experiments giving similar results.

compounds interfering with cellular ROS metabolism: ebselen, a scavenger of peroxynitrite, PEG-SOD, a cell permeant SOD, catalase which degrades H_2O_2 and, finally, stimgmatellin an inhibitor of the mitochondrial cytochrome bc1 complex. Among the different inhibitors applied, only PEG-SOD and stigmatellin exerted a clear inhibitory effect on PPIase inhibitor-induced ERK phosphorylation, thus indicating that mitochondrial superoxide generation is targeted by both PPIase inhibitors to trigger ROS formation (Fig. 4B).

3.5. Inhibition of metalloproteinase activity prevents ERK1/2 activation by PPIase inhibitors

The release of EGFR ligands by the action of metalloproteinases is a well-known mechanism for EGFR transactivation by G protein-coupled receptors [39,40]. Moreover, several reports have shown that the ectodomain shedding of HB-EGF in many cases depends on ADAM17, synonymously called TNFα converting enzyme (TACE) [26–28,41]. To evaluate the functional involvement of either of these proteases on PPIase inhibitor-induced ERK activation, we tested the broad spectrum MMP inhibitor GM6001 (100 nM), and TAPI-2 (100 μM) which preferentially inhibits ADAM17. As shown in Fig. 5 both compounds completely inhibited the PPIase inhibitor-induced ERK phosphorylation when applied 60 min prior to CsA or FK506.

3.6. ADAM17 is critical for ERK1/2 activation by PPIase inhibitors

To further test whether ADAM17 is critical for PPIase inhibitor-induced ERK activation, we silenced ADAM17 expression by transfection of specific siRNA. Concordant with the inhibitory effects of TAPI-2, genetic depletion of ADAM17 prevented constitutive and CNI-induced ERK1/2 phosphorylation (Fig. 6A). In addition, ERK phosphorylation itself seems to be activated by the transfection of siRNA as indicated by the high basal phosphorylated ERK1/2 content in unstimulated MC (Fig. 6A). In summary, this suggests that ADAM17 is critically involved in the CNI-induced but also in basal ERK1/2 activation in rat MC.

Next we tested whether the activity of ADAM17 itself is modulated by CNI by monitoring TACE substrate cleavage flurorimetrically. CsA and FK506 caused an almost three-fold increase in TACE activity when applied for 10 min (Fig. 6B). Importantly, the CNI-mediated increase in TACE activity was prevented when cells were cotreated with either SOD or with stigmatellin thus indicating that the increase in TACE activity by CNI critically depends on the generation of mitochondrial superoxide (Fig. 6B).

3.7. CNI-induced proliferation depends on ROS, ADAM17 and EGFR kinase activity

In a next step, we investigated whether the PPIase inhibitor-induced activation of the mitogenic ERK signaling cascade would functionally correlate with an increase in MC proliferation. For this purpose we labelled cells with BrdU. Serum starved MC were either treated with medium (control) or with different PPIase inhibitors (vehicle) for 24 h before cells were labelled with BrdU for additional 6 h. Interestingly, the DNA content of MC treated in the presence of CsA or FK506 showed an approx. 2.5-fold increase when compared with untreated control cells (Fig. 7A) demonstrating that CsA and

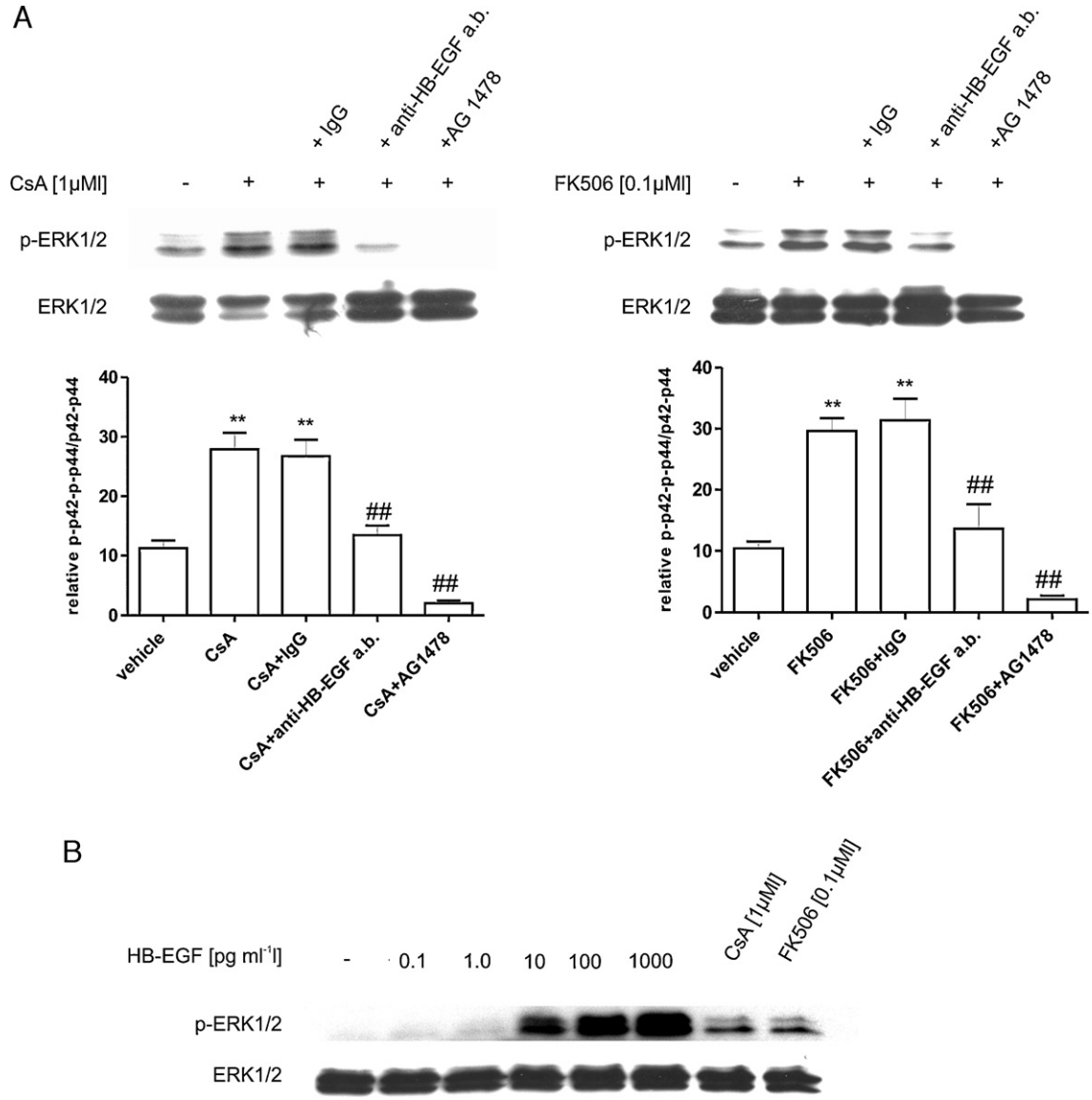

Fig. 3. PPIase inhibitor-induced ERK1/2 phosphorylation is impaired after neutralizing of HB-EGF. (A) Quiescent MC were pretreated for 60 min without or with either 20 μg of control IgG (+IgG), 20 μg of a neutralizing HB-EGF antibody (+anti-HB-EGF a.b.), or with AG1478 (250 nM). Thereafter, MC were stimulated for 10 min with either vehicle (–), CsA (left panel), or with 0.1 μM FK506 (right panel) as indicated. For Western blot analysis, 30 μg of total cell extracts was subjected to SDS-PAGE and successively probed with an anti-phospho-ERK1/2 antibody and antiserum against unphosphorylated ERK1/2. The data in the lower panels show a densitometrical analysis of phospho-ERK1/2 relative to total ERK1/2 levels and represent means ± S.D. ($n = 3$). $^{**}p \leq 0.01$ versus unstimulated control; and $\#\#p \leq 0.01$ versus PPIase inhibitor-stimulated conditions. (B) Dose-dependent activation of ERK1/2 by recombinant HB-EGF to estimate the levels of HB-EGF equipotent with ERK1/2 phosphorylation by PPIase inhibitors. Serum-deprived MC were treated with either vehicle (–) or with different amounts of recombinant HB-EGF, CsA (1 μM) or FK506 (0.1 μM) which all were applied for 10 min before cells were lyzed. Total protein lysate (30 μg) was subjected to Western blot analysis and successively probed with phospho-ERK1/2-specific and total ERK1/2-specific antibody. Data shown are representative of two independent experiments giving similar results.

FK506, both exert a strong mitogenic effect on MC. To further prove that the increase in cell proliferation evoked by the PPIase inhibitors relies on the postulated chain of signaling events initiated by CNI-induced ROS and followed by the ADAM17-dependent release of HB-EGF and subsequent activation of ERK, we tested whether inhibitors of these modules reduce the CNI-triggered increase in proliferation. To this end, MC were stimulated for 30 min with either NAC (5 mM), TAPI-2 (10 μM) or AG1478 (250 nM) before CsA or FK506 were applied for an additional 24 h. Interestingly, the CNI-induced cell proliferation was strongly reduced independent of which inhibitor was applied (Fig. 7A). Importantly, none of the inhibitors by themselves had any inhibitory effect on the basal proliferation rate of MC (Fig. 7B) nor had any significant effect on cell viability as monitored by MTT test (data not shown). Collectively, these data underscore the functional consequence of drug-induced ERK1/2 activation and, furthermore, support the permissive role of ROS, ADAM17 and EGFR activities in the proliferative effects of both PPIase inhibitors.

4. Discussion

Previously we have shown that the imunosuppressive drugs CsA and FK506, both in a concentration range which encompasses therapeutically relevant doses, can induce TGFβ-Smad signaling cascades in renal MC and thereby increase the expression of fibrogenic genes [17]. The current work extends those findings by demonstrating that CsA and FK506 in addition to the profibrotic TGFβ-Smad signaling cascade, similarly, can activate ERK1/2 signaling by a ROS-triggered mechanism. A redundancy of both signaling pathways is indicated by the observation that TGFβ itself

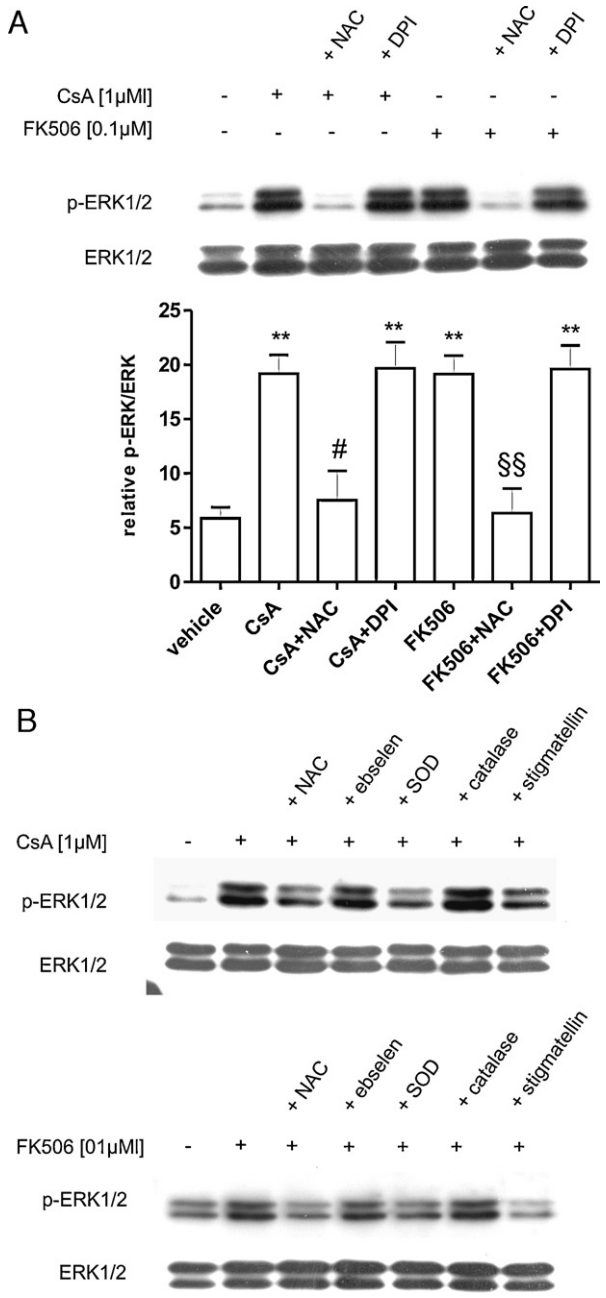

Fig. 4. PPIase inhibitor-mediated ERK1/2 activation depends on ROS. (A) Serum-starved MC were treated for 10 min with vehicle (–), CsA (1 μM), or FK506 (0.1 μM) in the absence or presence of either *N*-acetyl cysteine (NAC, 5 mM) or diphenylene iodonium (DPI, 10 μM) which were preincubated for 30 min. Subsequently, total cell lysates were assessed for phospho-ERK1/2 and total ERK1/2 contents. Data represent means ± S.D. ($n = 3$). $^{**}p \leq 0.01$ versus unstimulated control; $\#p \leq 0.05$ versus CsA-induced, §§$p \leq 0.01$ versus FK506-induced conditions. (B) 30 min prior to the addition of 1 μM CsA (upper panel) or 0.1 μM FK506 (lower panel), MC were treated without (–) or with different ROS scavengers including NAC (5 mM), ebselen (10 μM), PEG-SOD (SOD, 100 U/ml), catalase (500 U/ml), or stigmatellin (20 μM) for additional 40 min before cells were extracted for Western blot analysis. Total protein (30 μg) was subjected to SDS-PAGE and blots were successfully probed with anti-phospho-ERK1/2-specific and anti-ERK1/2-specific antibodies. Data shown are representative of two independent experiments giving similar results.

is able to activate ERK and its downstream signaling members [19,20]. Additionally, a previous study proved the existence of a cross-talk between ERK and TGFβ-Smad signaling pathways which is functionally relevant for increased collagen production in human MC [21]. However, in rat MC, a similar cross activation of ERK by TGFβ seems not relevant for PPIase inhibitor-induced

Table 1
Effects of different ROS scavengers on CNI-induced radical formation. Quiescent MC were treated for 60 min with vehicle, CsA (1 μM), or FK506 (0.1 μM) in the presence of either NAC (5 mM) or DPI (10 μM) before cell supernatants were assayed for ROS which was measured by dichlorofluorescein (DCF) formation. Treatment-dependent DCF formation is expressed as arbitrary units. Data are means ± S.D. ($n = 3$) with all experiments were done in triplicates n.s.: not significant.

Treatment	DCF (arbitrary units)	$p(\leq)$ *vs* vehicle (*)/*vs* CNI-induction (#)
Vehicle	14,756 ± 7700	–
CsA	33,704 ± 6799	0.01 (*)
CsA + NAC	17,854 ± 4378	n.s. (*)/0.05 (#)
CsA + DPI	28,737 ± 4789	0.05 (*)/n.s. (#)
FK506	46,868 ± 9678	0.01 (*)
FK506 + NAC	13,209 ± 5672	n.s. (*)/0.05 (#)
FK506 + DPI	37,432 ± 9866	0.05 (*)/n.s. (#)

ERK signaling since an incubation with a neutralizing TGFβ antiserum did not attenuate the drug-induced ERK1/2 phosphorylation (data not shown). In the course of glomerulonephritis, ERK-dependent proliferation of MC is relevant for maintenance or exacerbation of ECM accumulation, a key feature for the development of renal sclerosis [42]. It is interesting to note that the temporal activation of both pathways differs since the activation of ERK in many cases precedes a phase of ECM deposition as observed in the course of various progressive renal diseases [34,35]. Here we present mechanistic relevance of a

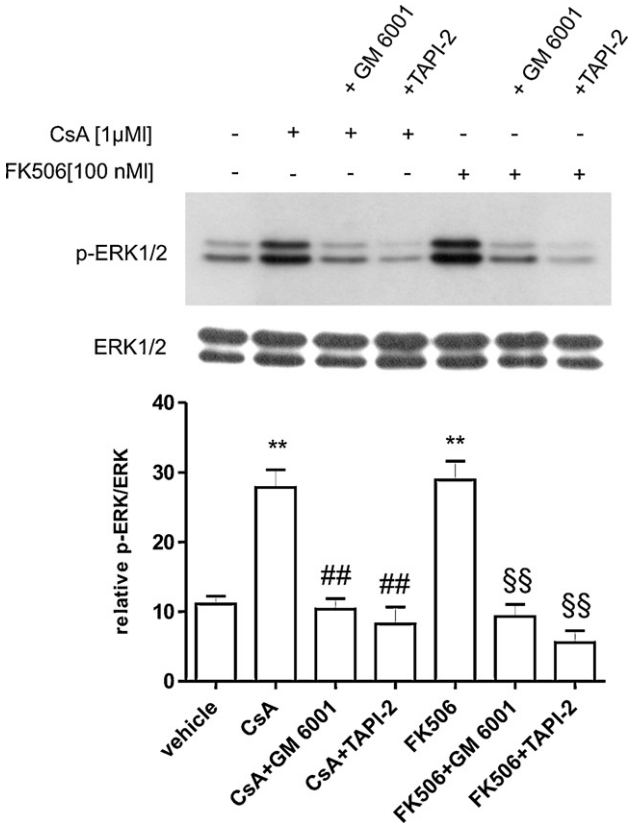

Fig. 5. Metalloproteinase activity is critical for ERK1/2 activation by CsA and FK506. Serum deprived MC were treated for 10 min with vehicle (–), CsA (1 μM), or FK506 (0.1 μM) in the absence or presence of the broad spectrum MMP inhibitor GM6001 (10 μM), or TAPI-2 (10 μM), a MMP inhibitor with a high specificity towards ADAM17. Both inhibitors were preincubated for 30 min before the indicated PPIase inhibitor was added. Subsequently, cells were harvested for total cell lysates. For Western blot analysis, the blots were successfully probed with anti-phospho-ERK1/2-specific and anti-ERK1/2-specific antibodies. Data represent means ± S.D. ($n = 3$).$^{**}p \leq 0.01$ versus vehicle (–); ##$p \leq 0.01$ versus CsA-induced; §§$p \leq 0.01$ versus FK506-induced conditions.

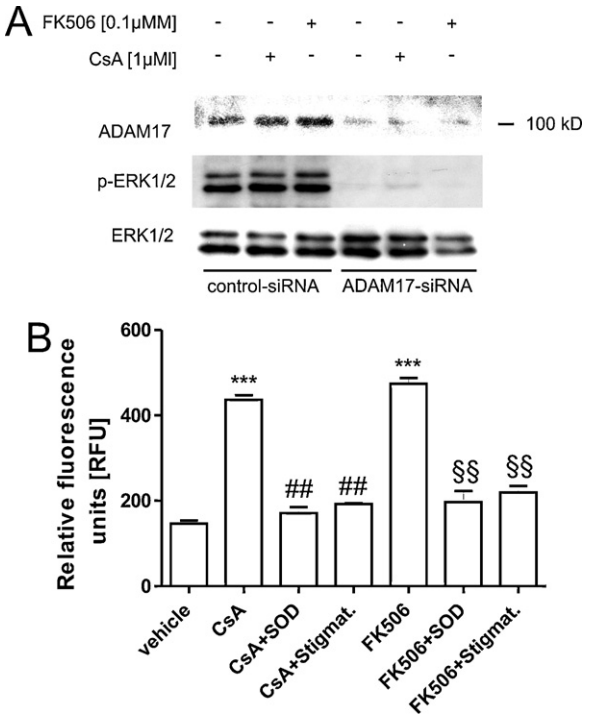

Fig. 6. The constitutive and PPIase inhibitor-induced ERK1/2 activity depends on ADAM17. (A) MC were transfected with siRNA duplexes of rat-specific ADAM17 (ADAM17-siRNA), or with a none-gene-related control siRNA (control-siRNA). After transfection, MC were serum deprived for 16 h and subsequently treated with either vehicle (–), CsA (1 μM), or FK506 (0.1 μM) for 10 min before cells were harvested for total protein extraction. To prove a knockdown of ADAM17, 30 μg of protein extract was probed with an ADAM17-specific antibody by Western blot analysis as indicated. In parallel, 30 μg of protein extracts were additionally probed with a phospho-ERK1/2-specific and with an antibody raised against total ERK1/2. The data shown are representative of two independent experiments giving similar results. (B) PPIase inhibitor-induced fluorogenic TACE substrate depends on mitochondrial ROS. Serum-starved MC were stimulated for 10 min with either vehicle, CsA (1 μM), or FK506 (0.1 μM). Both immunosuppressants were applied either without, or with the indicated ROS scavengers which both were preincubated for 30 min. Subsequently, cell lysates were prepared and TACE activity was measured by using the flurometric *SensoLyteR TACE-activity assay kit* (ANASPEC). Enzymatic activity was assessed by measuring changes in relative fluorescence (RFU). Data represent means ± S.D. ($n = 3$) of triplicate experiments; $^{***}p \leq 0.005$ versus vehicle; $\#\#p \leq 0.01$ versus CsA-induced; $\S\S p \leq 0.01$ versus FK506-induced conditions.

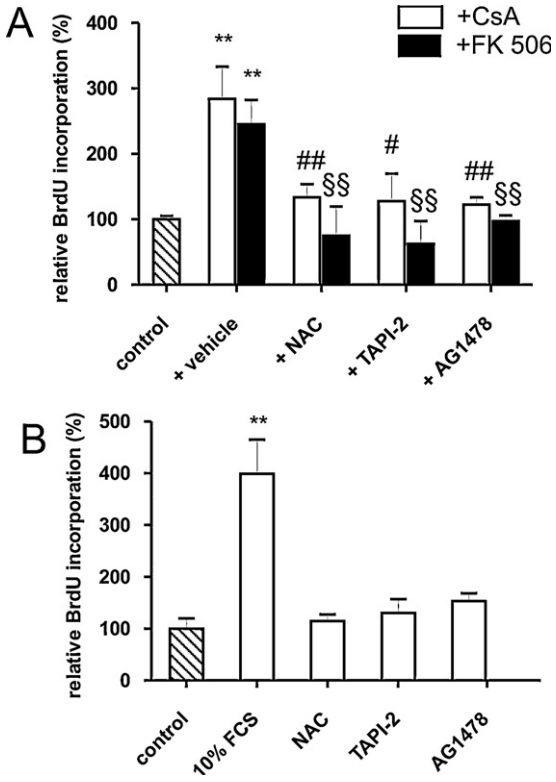

Fig. 7. Stimulation with CsA and FK506 leads to an increase in DNA content. (A) Serum-starved MC were treated without (control) or with CsA (1 μM), or FK506 (0.1 μM) in the absence (vehicle) or presence of the indicated inhibitors NAC (5 mM), TAPI-2 (10 μM) or AG1478 (250 nM) for 24 h before cells were labelled with BrdU for an additional 12 h. (B). Serum-starved MC were treated without (control) or with 10% FCS used as a positive control, or alternatively, with one of the indicated inhibitors for 24 h before cells were labelled with BrdU for an additional 12 h. The concentrations used were similar as described in (A). Cell proliferation was assessed by detection of BrdU incorporation into DNA and is depicted as relative BrdU incorporation (%) in comparison with unstimulated cells which were set to 100%. Results represent means ± S.D. ($n = 3$). $^{**}p \leq 0.01$ versus control; $\#p \leq 0.05$, $\#\#p \leq 0.01$ versus CsA-induced (open bars); $\S\S p \leq 0.01$ versus FK506-induced conditions (black bars).

signaling cascade involving ROS-ADAM17-HB-EGF-EGFR-ERK that contributes to CsA or FK506-mediated increase in mesangial cell proliferation. A functional involvement of HB-EGF-mediated EGFR signaling is indicated by the strong inhibitory effects of a neutralizing HB-EGF antibody and by AG1478, a highly potent inhibitor of the EGFR tyrosine kinase. The functional role of HB-EGF is furthermore highlighted by the fact that induction of ERK phosphorylation is mimicked by exogenously administered HB-EGF (Fig. 3B). Various studies have demonstrated that in renal MC several endogenous mediators, including aldosterone [43], 5-hydroxytryptamine [27], TGFβ [20] and glucose [26] commonly transactivate the EGFR. Interestingly, the mTOR inhibitor rapamycin, which induces a similar profibrotic cell response in MC [44], can increase the survival of different cell types via EGFR-ERK1/2 signaling, by an involvement of c-Src but independent of EGF or EGF-related growth factors [45].

Furthermore, our data provide evidence that ADAM17 plays an important role in the activation of ERK1/2 by both tested PPIase inhibitors as indicated by the strong inhibitory effects by genetic or pharmacological ADAM17 inhibition (Figs. 5 and 6A). Correspondingly, TACE activity is significantly increased by CsA and FK506 (Fig. 6B) suggesting that the PPIase inhibitor-induced activation of ERK involves an ADAM17-dependent release of HB-EGF. Our data are in accordance with studies demonstrating that the transactivation of EGFR by either G protein-coupled receptors or oxidative and osmotic stress strongly depends on MMP-mediated cleavage of HB-EGF [20,27,39–41]. Moreover, an oxidant-induced increase in ADAM17 activity and a subsequent HB-EGF ectodomain shedding has been reported for the PKC inhibitor chelerythrin [33]. In line with those studies, the activation of TACE by PPIase inhibitors observed in MC is ROS-dependent implicating that ROS acts upstream of HB-EGF. It is worth mentioning that ADAM17 is not only critical for the transactivation of EGFR by HB-EGF [41] but has been shown to be involved in EGFR transactivation by TGFα which seems functionally relevant for the increased intestinal permeability in response to oxidative stress [46]. An activation of ERK1/2 is also implied in the control of renal epithelial barrier function and of high relevance for CsA-induced disturbance of transepithelial resistance [47]. In light of these observations, it is tempting to speculate that PPIase inhibitor-induced activation of HB-EGF-EGFR signaling is operative in other renal cell types and an important feature of nephrotoxicity induced by both immunosuppressive drugs. Since ectodomain shedding by metalloproteinases or ADAMs is considered a central mechanism of ligand-dependent activation of EGFR, the contribution of ROS-mediated EGFR activation may critically depend on the spatiotemporal presence and release of different EGFR ligands. Further investigations are

needed to test whether ligands other than HB-EGF are involved in the activation of EGFR-induced mitogenic ERK signaling by CsA and FK506.

Of note, a previous study in rat MC has demonstrated that both PPIase inhibitors can rapidly induce the cold-shock protein Y-box binding protein 1 (YB-1) by a sequence of consecutive processes which include ROS generation and subsequent activation of ERK/Akt pathways [18]. The PPIase inhibitor-induced activation of the mRNA-binding protein YB-1 results in an accumulation of fibrogenic genes mainly via posttranscriptional mechanisms [18]. In contrast to the latter report, we obtained evidence that ROS production by both immunosuppressants was not affected by catalase thus excluding a critical involvement of H_2O_2 in the drug-induced ERK activation in MC (Fig. 4B). However, the cell permeant PEG-SOD and stigmatellin caused a marked attenuation in PPIase inhibitor-induced ERK activity indicating that mitochondrial enzymes may represent the main sources of PPIase inhibitor-triggered ROS formation in the kidney (Fig. 4B). Our observations are corroborated by results obtained in smooth muscle cells, demonstrating that CsA induced ROS generation independent of cytochrome P-450 oxidases and NADPH oxidases [13,14]. Obviously, in MC both immunosuppressants can induce profibrotic and mitogenic responses by simultaneously increasing the expression and/or activity of different ROS generating enzymes.

A further challenging issue which needs to be addressed by future experiments is the elucidation of the mechanism underlying ROS-mediated HB-EGF shedding by PPIase inhibitors. In fact, an activation of MMP-dependent HB-EGF release by oxidative stress has already been demonstrated in several previous studies [33,41,46]. Depending on the cell type investigated, the activation of MMPs or ADAMs can either occur by a direct ROS effect or, indirectly, through the action of a protein which is upregulated by ROS. Activation of the ERK pathway plays a key role in tubular epithelial cell proliferation in the injured kidney as demonstrated in a rat model of unilateral ureteral obstruction [48]. In addition, an increase in ROS formation is responsible for CsA-induced cytotoxicity described with high doses of CsA [12,49,50]. However, consistent with our data, studies in mouse tubular epithelial cells have demonstrated that the ERK pathway itself is not critical for CsA toxicity since pharmacological inhibitors of MEK did not prevent CsA-mediated cytotoxicity [51]. Irrespective of whether ROS are critical for PPIase inhibitor-mediated cytotoxicity, the doses of CsA and FK506 used in our experiments did not significantly affect viability of MC [52].

In conclusion, the convergent activation of profibrotic and mitogenic cell responses by CsA and FK506 emphasizes the complex repertoire of regulatory events triggered by these widely used drugs. Definitely, our data provided here warrant further experiments exploring the pathophysiological relevance of these mechanisms in PPIase inhibitor-induced nephropathy.

Acknowledgements

We thank Roswitha Müller and Analena Henrich for excellent technical assistance. This work was supported by the German Research Foundation (DFG) grants, EB 257/5-1, PF 361/7-1, FOG 784, GRK1172, SFB 815 and Excellence Cluster "Cardiopulmonary System" (ECCPS) EXC 147/1.

References

[1] Eddy AA. Molecular insights into renal interstitial fibrosis. J Am Soc Nephrol 1996;7:2495–508.
[2] Eddy AA, Fogo AB. Plasminogen activator inhibitor-1 in chronic kidney diseases: evidence and mechanisms of action. J Am Soc Nephrol 2006;17:2999–3012.
[3] Kashgarian M, Sterzel RB. The pathobiology of the mesangium. Kidney Int 1992;41:524–9.
[4] Pfeilschifter J. Mesangial cells orchestrate inflammation in the renal glomerulus. News Physiol Sci 1994;9:271–6.
[5] Mihatsch MJ, Kyo M, Morozumi K, Yamaguchi Y, Nickeleit V, Ryffel B. The side-effects of cyclosporin A and tacrolimus. Clin Nephrol 1998;49:356–63.
[6] Khanna AK, Cairns VR, Becker CG, Hosenpud JD. Transforming growth factor (TGF)-β mimics and anti-TGF-β antibody abrogates the in vivo effects of cyclosporine: demonstration of a direct role of TGF-β in immunosuppression and nephrotoxicity of cyclosporine. Transplantation 1999;67:882–9.
[7] Ziyadeh FN, Hoffman BB, Han DC, Iglesias-De La Cruz MC, Hong SW, Isono M, et al. Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-β antibody in db/db diabetic mice. Proc Natl Acad Sci U S A 2000;97:8015–20.
[8] Islam M, Burke JFJr, McGowan TA, Zhu Y, Dunn SR, McCue P, et al. Effect of anti-transforming growth factor-beta antibodies in cyclosporine-induced renal dysfunction. Kidney Int 2001;59:498–506.
[9] Ling H, Li X, Jha S, Wang W, Karetskaya L, Pratt B, et al. Therapeutic role of TGF-β-neutralizing antibody in mouse cyclosporin A nephropathy: morphologic improvement associated with functional preservation. J Am Soc Nephrol 2003;4:377–88.
[10] Fukasawa H, Yamamoto T, Suzuki H, Togawa A, Ohashi N, Fujigaki Y, et al. Treatment with anti-TGF-β antibody ameliorates chronic progressive nephritis by inhibiting Smad/TGF-β signaling. Kidney Int 2004;65:63–74.
[11] Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK506. Immunol Today 1992;13:136–42.
[12] Wang C, Salahudeen AK. Cyclosporine nephrotoxicity: attenuation by an antioxidant-inhibitor of lipid peroxidation in vitro and in vivo. Kidney Int 1994;47:927–34.
[13] Krauskopf A, Buetler TM, Nguyen NSD, Macé K, Ruegg UT. Cyclosporin A-induced free radical generation is not mediated by cytochrome P-450. Br J Pharmacol 2002;135:977–86.
[14] Krauskopf A, Lhote P, Petermann O, Ruegg UT, Buetler TM. Cyclosporin A generates superoxide in smooth muscle cells. Free Radic Res 2005;39:913–9.
[15] Ichikawa I, Kiyama S, Yoshioka T. Renal antioxidant enzymes: their regulation and function. Kidney Int 1994;45:1–9.
[16] Rezzani R. Renal antioxidant enzymes: their regulation and function. Histol Histopathol 2006;21:301–16.
[17] Akool el-S, Doller A, Babelova A, Tsalastra W, Moreth K, Schaefer L, et al. Molecular mechanisms of TGF β receptor-triggered signaling cascades rapidly induced by the calcineurin inhibitors cyclosporin A and FK506. J Immunol 2008;181:2831–45.
[18] Hanssen L, Frye BC, Ostendorf T, Alidousty C, Djudjaj S, Boor P, et al. Y-box binding protein-1 mediates profibrotic effects of calcineurin inhibitors in the kidney. J Immunol 2011;187:298–308.
[19] Yan Z, Winawer S, Friedman E. Two different signal transduction pathways can be activated by transforming growth factor β 1 in epithelial cells. J Biol Chem 1994;269:13231–7.
[20] Uchiyama-Tanaka Y, Matsubara H, Mori Y, Kosaki A, Kishimoto N, Amano K, et al. Involvement of HB-EGF and EGF receptor transactivation in TGF-β-mediated fibronectin expression in mesangial cells. Kidney Int 2002;62:799–808.
[21] Hayashida T, Decaestecker M, Schnaper HW. Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF-β-dependent responses in human mesangial cells. FASEB J 2003;17:1576–8.
[22] Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 2000;19:3159–67.
[23] Schneider MR, Wolf E. The epidermal growth factor receptor ligands at a glance. J Cell Physiol 2009;218:460–6.
[24] Holbro T, Hynes NE. ErbB receptors: directing key signaling networks throughout life. Annu Rev Pharmacol Toxicol 2004;44:195–217.
[25] Takemura T, Murata Y, Hino S, Okada M, Yanagida H, Ikeda M, et al. Heparin-binding EGF-like growth factor is expressed by mesangial cells and is involved in mesangial proliferation in glomerulonephritis. J Pathol 1999;189:431–8.
[26] Uttarwar L, Peng F, Wu D, Kumar S, Gao B, Ingram AJ, et al. HB-EGF release mediates glucose-induced activation of the epidermal growth factor receptor in mesangial cells. Am J Physiol Renal Physiol 2011;300:F921–31.
[27] Göoz M, Göoz P, Luttrell LM, Raymond JR. 5-HT2A receptor induces ERK phosphorylation and proliferation through ADAM-17 tumor necrosis factor-alpha-converting enzyme (TACE) activation and heparin-bound epidermal growth factor-like growth factor (HB-EGF) shedding in mesangial cells. J Biol Chem 2006;281:21004–12.
[28] Kveiborg M, Instrell R, Rowlands C, Howell M, Parker PJ. PKCα and PKCδ regulate ADAM17-mediated ectodomain shedding of heparin binding-EGF through separate pathways. PLoS One 2011;6:e17168.
[29] Gechtman Z, Alonso JL, Raab G, Ingber DE, Klagsbrun M. The shedding of membrane-anchored heparin-binding epidermal-like growth factor is regulated by the Raf/mitogen-activated protein kinase cascade and by cell adhesion and spreading. J Biol Chem 1999;274:28828–35.
[30] Kenny PA, Bissell MJ. Targeting TACE-dependent EGFR ligand shedding in breast cancer. J Clin Invest 2007;117:337–45.
[31] Higashiyama S, Nanba D. ADAM-mediated ectodomain shedding of HB-EGF in receptor cross-talk. Biochim Biophys Acta 2005;1751:110–7.

[32] Huovila AP, Turner AJ, Pelto-Huikko M, Kärkkäinen I, Ortiz RM. Shedding light on ADAM metalloproteinases. Trends Biochem Sci 2005;30:413–22.
[33] Kim J, Lin J, Adam RM, Lamb C, Shively SB, Freeman MR. An oxidative stress mechanism mediates chelerythrine-induced heparin-binding EGF-like growth factor ectodomain shedding. J Cell Biochem 2005;94:39–49.
[34] Floege J, Burns MW, Alpers CE, Yoshimura A, Pritzl P, Gordon K, et al. Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. Kidney Int 1992;41:297–309.
[35] Bessho K, Mizuno S, Matsumoto K, Nakamura T. Counteractive effects of HGF on PDGF-induced mesangial cell proliferation in a rat model of glomerulonephritis. Am J Physiol Renal Physiol 2003;284:F1171–80.
[36] Pfeilschifter J, Vosbeck K. Transforming growth factor β 2 inhibits interleukin 1 β- and tumour necrosis factor alpha-induction of nitric oxide synthase in rat renal mesangial cells. Biochem Biophys Res Commun 1991;175:372–9.
[37] Eberhardt W, Huwiler A, Beck KF, Walpen S, Pfeilschifter J. Amplification of IL-1 β-induced matrix metalloproteinase-9 expression by superoxide in rat glomerular mesangial cells is mediated by increased activities of NF-kappa B and activating protein-1 and involves activation of the mitogen-activated protein kinase pathways. J Immunol 2000;165:5788–97.
[38] Huwiler A, Akool el-S, Aschrafi A, Hamada FM, Pfeilschifter J, Eberhardt W. ATP potentiates interleukin-1 β-induced MMP-9 expression in mesangial cells via recruitment of the ELAV protein HuR. J Biol Chem 2003;278:51758–69.
[39] Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, et al. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 1999;402:884–8.
[40] Ohtsu H, Dempsey PJ, Eguchi S. ADAMs as mediators of EGF receptor transactivation by G protein-coupled receptors. Am J Physiol Cell Physiol 2006;291:C1–0.
[41] Fischer OM, Hart S, Gschwind A, Prenzel N, Ullrich A. Oxidative and osmotic stress signaling in tumor cells is mediated by ADAM proteases and heparin-binding epidermal growth factor. Mol Cell Biol 2004;24:5172–83.
[42] Floege J, Eng E, Young BA, Couser WG, Johnson RJ. Heparin suppresses mesangial cell proliferation and matrix expansion in experimental mesangioproliferative glomerulonephritis. Kidney Int 1993;43:369–80.
[43] Huang S, Zhang A, Ding G, Chen R. Aldosterone-induced mesangial cell proliferation is mediated by EGF receptor transactivation. Am J Physiol Renal Physiol 2009;296:F1323–3.
[44] Osman B, Doller A, Akool el-S, Holdener M, Hintermann E, Pfeilschifter J, et al. Rapamycin induces the TGFβ1/Smad signaling cascade in renal mesangial cells upstream of mTOR. Cell Signal 2009;21:1806–17.
[45] Chaturvedi D, Gao X, Cohen MS, Taunton J, Patel TB. Rapamycin induces transactivation of the EGFR and increases cell survival. Oncogene 2009;28:1187–96.
[46] Forsyth CB, Banan A, Farhadi A, Fields JZ, Tang Y, Shaikh M, et al. Regulation of oxidant-induced intestinal permeability by metalloprotease-dependent epidermal growth factor receptor signaling. J Pharmacol Exp Ther 2007;321:84–97.
[47] Kiely B, Feldman G, Ryan MP. Modulation of renal epithelial barrier function by mitogen-activated protein kinases (MAPKs): mechanism of cyclosporin A-induced increase in transepithelial resistance. Kidney Int 2003;63:908–16.
[48] Masaki T, Foti R, Hill PA, Ikezumi Y, Atkins RC, Nikolic-Paterson DJ. Activation of the ERK pathway precedes tubular proliferation in the obstructed rat kidney. Kidney Int 2003;63:1256–64.
[49] Wolf A, Trendelenburg CF, Diez-Fernandez C, Prieto P, Houy S, Trommer WE, et al. Cyclosporin A-induced oxidative stress in rat hepatocytes. J Pharmacol Exp Ther 1997;280:1328–34.
[50] Burdmann EA, Andoh TF, Yu L, Bennett WM. Cyclosporine nephrotoxicity. Semin Nephrol 2003;23:465–76.
[51] Sarró E, Tornavaca O, Plana M, Meseguer A, Itarte E. Phosphoinositide 3-kinase inhibitors protect mouse kidney cells from cyclosporine-induced cell death. Kidney Int 2008;73:77–85.
[52] Doller A, Akool el-S, Müller R, Gutwein P, Kurowski C, Pfeilschifter J, et al. Molecular mechanisms of cyclosporin A inhibition of the cytokine-induced matrix metalloproteinase-9 in glomerular mesangial cells. J Am Soc Nephrol 2007;18:581–92.