TGF-β1 induces COX-2 expression and PGE_2 synthesis through MAPK and PI3K pathways in human mesangial cells

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Transforming growth factor-β1 (TGF-β1) plays a fundamental role in the progression of renal diseases. Accumulating evidence has suggested that eicosanoids derived from cyclooxygenase-2 (COX-2) participate in a number of pathological processes in immune-mediated renal diseases. Mesangial cells (MC) play a major role in physiological and pathophysiological renal processes. MC express receptors for TGF-β1, and COX-2 expression can be induced in MC. However, to date, there are no published data on the possible role of TGF-β1 in COX-2 expression in human mesangial cells (HMC). We designed studies to determine (1) whether TGF-β1 stimulates COX-2 expression in primary HMC, (2) whether mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) cascades are involved in TGF-β1-induced COX-2 expression, and (3) whether prostaglandin (PG)E_2 synthesis is affected by TGF-β1 and MAP kinases and PI3K activation. Studies were performed in primary cultures of HMC and in an immortalized line of HMC. TGF-β1 induces COX-2 promoter activity and COX-2 mRNA and protein expression in HMC. COX-2 induction is accompanied by increased PGE_2 synthesis. Extracellular signal-regulated kinase (ERK)1/2, p38 MAPK, and PI3K pathway inhibition blunted TGF-β1-induced COX-2 overexpression. We demonstrate that TGF-β1 regulates COX-2 expression in HMC through the activation of ERK1/2, p38 MAPK, and PI3K. These results can help to elucidate the molecular mechanisms underlying the regulation of COX-2 and open up specific strategies for the treatment of glomerular disease.

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Glomerular mesangial cells (MC) play a key role in glomerular sclerosis and in the maintenance of immune functions of the glomeruli.[1] MC damage may lead to a serious deterioration of glomerular functions. Accumulating evidence has suggested that specific eicosanoids participate in a number of pathological processes leading to end-stage renal disease.[2] Cyclooxygenase (COX), also known as prostaglandin (PG)G/H synthase, is a membrane-bound, bifunctional enzyme that catalyzes the conversion of arachidonic acid to PGG_2 by its COX activity and of PGG_2 to PGH_2 by peroxidase activity. It is the rate-limiting step in the biosynthesis of biologically active and physiologically important PGs.[3] Two isoforms of COX have been identified, COX-1 and COX-2.[4] COX-1 is constitutively expressed in most tissues. In contrast, COX-2 operates as an inducible enzyme with low or undetectable levels in most tissues.[5] COX-2 expression is induced by cytokines, tumour promoters, and growth factors (reviewed by Williams and DuBois[4] and Kramer *et al.*[6]). Increased renal expression of COX-2 has been reported in nephropathies such as renal failure,[6] diabetic nephropathy,[7] and systemic lupus erythematosus.[8] However, some of the pathways involved in the mesangial synthesis of PG via COX-2 as well the biologic consequences of glomerular PG synthesis remain poorly understood.

COX-2 expression is regulated by multiple signalling pathways and its regulation is cell type and stimulus dependent.[9] Among the mechanisms involved in the regulation of COX-2 expression, the activation of small Guanine triphosphatase ras[10,11] and several mitogen-activated protein kinases (MAPK)[12] seems to play a major role.

Extensive studies have demonstrated that transforming growth factor-β (TGF-β) plays an important role in the progression of renal diseases.[13] TGF-β1 has been linked mainly to fibrogenesis in experimental models of glomerulonephritis and diabetic nephropathy.[14] Interestingly, one of the remarkable effects of TGF-β1 is the induction of COX-2 expression.[15–17] However, there are no data on the possible effect of TGF-β on COX-2 expression in MC. Based upon the observations that COX-2 is expressed in kidney diseases and

in MC[6] and that TGF-β1 is overexpressed in renal fibrosis and in stimulated MC,[18] we hypothesized that TGF-β1 may play a role in the regulation of COX-2 expression in human mesangial cells (HMC). Thus, a major purpose of this study was to assess whether TGF-β1 stimulates COX-2 expression in primary cultured HMC.

In the present study, we demonstrate for the first time that exogenous TGF-β1 induces COX-2 promoter activity and COX-2 mRNA and protein expression in HMC. In addition, we demonstrate that TGF-β1 regulates COX-2 expression in HMC through several signalling routes including activation of p38, extracellular signal-regulated kinase (ERK)1/2, and Akt.

RESULTS

TGF-β1 induces COX-2 expression in HMC

The first aim of this study was to determine whether TGF-β1 induced COX-2 expression in primary HMC. For this purpose, proteins from cells extracts treated with TGF-β1 were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting using an anti-COX-2 antibody. Subconfluent serum-starved HMC expressed relatively low levels of COX-2 protein (Figure 1a). Stimulation of these cells with TGF-β1 (1 ng/ml) induced a marked increase in COX-2 protein, which was evident within 4 h after agonist treatment, reached a maximum at 24 h, and the increase remained detectable for up to 48 h (Figure 1a). COX-2 protein levels were also measured in cells treated with increasing amounts of TGF-β1 (0.1, 1, and 10 ng/ml) for 24 h. A concentration-dependent increase in COX-2 expression was observed (Figure 1b). COX-1 was constitutively expressed and did not change after TGF-β1 treatment (Figure 1a and b).

We next assessed whether the TGF-β1-induced increase in COX-2 expression has a functional consequence such as an increase in prostanoids production. PGE_2 is a major product of COX-initiated arachidonic acid metabolism in MC.[19] We found that TGF-β1 at 1 ng/ml induced PGE_2 production in a time-dependent manner. TGF-β1-induced PGE_2 generation was increased after 4 h, and the highest PGE_2 concentration was achieved at 24–48 h of stimulation (Figure 2a). The time course of TGF-β1-induced increase in PGE_2 production was similar to that for COX-2 expression. PGE_2 concentration was corrected by the protein content in each culture plate. These data suggest that the increase in COX-2 expression is functional. To verify whether the increase in PGE_2 production induced by TGF-β1 is based on COX-2 increase, HMC were pretreated with the specific inhibitor of COX-2 activity NS-398 (10 μM). This substance blocked TGF-β1-induced PGE_2 production but not TGF-β1-induced COX-2 expression (Figure 2b). These results demonstrate that the increase in PGE_2 production induced by TGF-β1 is mediated by upregulation of COX-2 expression.

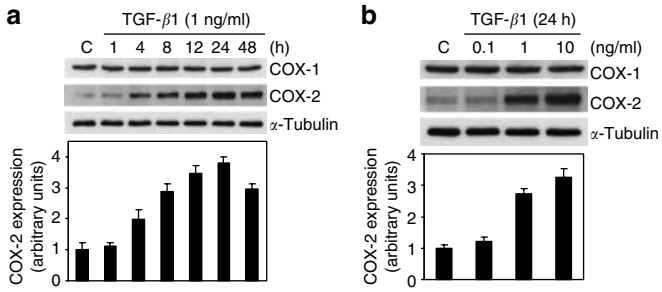

Figure 1 | Time- and dose-dependent induction of COX-2 by TGF-β1. (**a**) HMC were serum-starved for 24 h and treated with TGF-β1 (1 ng/ml) for the indicated time points. Total protein lysates were analyzed by Western blotting using anti COX-1 and COX-2 antibodies. Equal amounts of protein were loaded in each lane, which was verified by blotting with anti-α-tubulin. (**b**) Serum-starved HMC were treated with increasing amounts of TGF-β1 (0.1–10 ng/ml) for 24 h. Cells were lysed and COX-1 and COX-2 expression was analyzed by Western blotting. Results are expressed as the mean ± s.e.m. of, at least, two independent experiments.

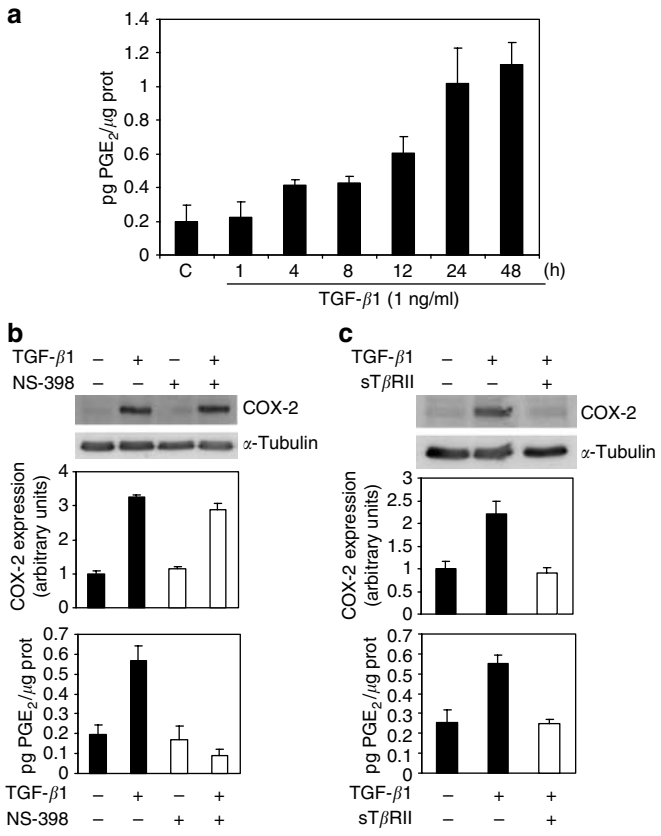

Figure 2 | TGF-β1 induces COX-2-dependent PGE_2 production. (**a**) HMC were serum-starved (24 h) before stimulation with TGF-β1 (1 ng/ml) and PGE_2 ELISA analysis was performed in the culture medium. PGE_2 levels were corrected by protein amount present in each plate. (**b**) Serum-starved HMC were untreated or treated with the specific COX-2 inhibitor NS-398 (10 μM) 1 h before incubation with TGF-β1 (1 ng/ml) for 24 h. Protein extracts were analyzed by Western blotting for COX-2 expression. The culture medium was collected to measure PGE_2 by ELISA. (**c**) Serum-starved HMC were untreated or treated with the sTβRII (500 ng/ml) and/or with TGF-β1 (1 ng/ml) for 24 h. Protein extracts were analyzed by Western blotting for COX-2 expression. The culture medium was collected to measure PGE_2 by ELISA. Results are expressed as the mean ± s.e.m. of, at least, two independent experiments.

To determine the specificity of TGF-β1-dependent induction of COX-2 and PGE_2, HMC were pretreated with the soluble TGF-β receptor II (sTβRII) (500 ng/ml) before treatment with TGF-β1 (1 ng/ml) for 24 h. Soluble TGF-β1 receptor II inhibited both the TGF-β1-induced COX-2 expression and the increased PGE_2 release (Figure 2c).

TGF-β1-dependent COX-2 expression involves transcriptional mechanisms

COX-2 expression is highly regulated by both transcriptional and post-transcriptional mechanisms, depending on its activator and the cell type.[20,21] Thus, our next purpose was to determine whether TGF-β1 increases COX-2 gene expression in HMC. For this purpose, total RNA was isolated from cells treated with TGF-β1 (1 ng/ml) for various time points (1–48 h). The levels of COX-2 mRNA were measured by reverse transcription-polymerase chain reaction (RT-PCR). TGF-β1 induced a time-dependent increase in COX-2 mRNA levels, which peaked at 12 h after addition of the agonist (Figure 3a). PCR was also performed for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA to control for the PCR efficiency and equal starting amount of total RNA. There was no apparent change in GAPDH mRNA abundance upon TGF-β1 treatment. As stimulation of HMC resulted in increased expression of the COX-2 mRNA, we next aimed to assess changes in COX-2 promoter activity in response to TGF-β1. HMC were transfected with a luciferase reporter vector containing an exogenous COX-2 promoter[22] and then stimulated with TGF-β1 (1 ng/ml) for 12 h (Figure 3b). Our results demonstrated that incubation with TGF-β1 resulted in an increased activity of the COX-2 promoter.

As post-transcriptional regulation of COX-2 expression includes changes in COX-2 mRNA stability, we examined the role of a post-transcriptional mechanism involving mRNA stabilization. To address this possibility, we employed a classical technique involving the measurement of COX-2 mRNA levels in transcriptionally arrested cells using actinomycin D (Act D) in the absence or presence of TGF-β1 (Figure 3c). HMC were treated with TGF-β1 (1 ng/ml) for 12 h (maximum mRNA level; see Figure 3a). At this time point (0 h), we distinguished three conditions. In the first two conditions, the medium already containing TGF-β1 was retained for another 0.5, 1, and 2 h. In the third condition, the medium was replaced with fresh medium without TGF-β1. The transcriptional inhibitor Act D (5 μg/ml) was added to the plates of the second and third conditions. Total RNA was isolated at the indicated time points and examined for the presence of COX-2 mRNA by RT-PCR. To correct differences in loading, the signal density of each COX-2 band was divided by the signal density of the GAPDH band. As shown in Figure 3c, we observed that TGF-β1-induced COX-2 mRNA levels remained relatively stable in non-Act D-treated samples; however, after Act D halted transcription, there was a decrease in COX-2 mRNA expression. The COX-2 mRNA levels dropped in the presence of Act D with a similar slope in the presence or absence of TGF-β1, indicating that transcriptional activity, rather than increased mRNA stability, is the primary mechanism responsible for TGF-β1-induced increase in COX-2 mRNA levels.

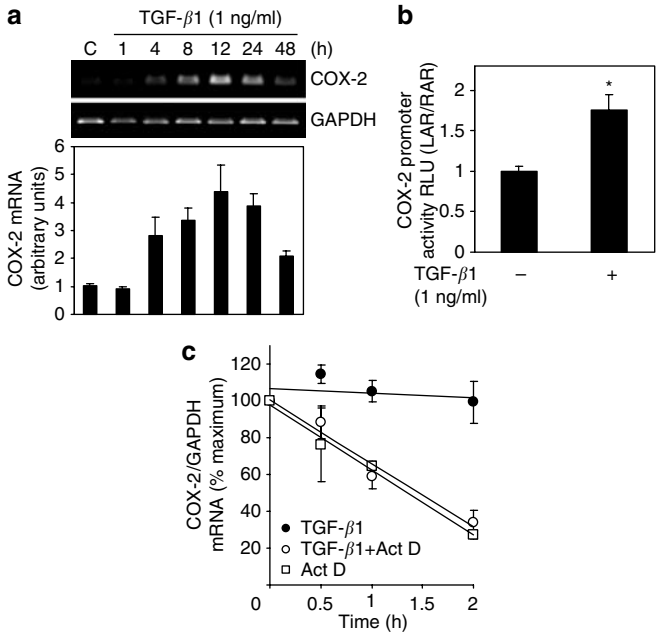

Figure 3 | COX-2 expression by TGF-β1 involves transcriptional mechanisms. (**a**) HMC were serum-starved (24 h) before stimulation with TGF-β1 (1 ng/ml). RNA was isolated at the indicated time points after addition of agonist and COX-2 mRNA was analyzed by RT-PCR. The expression of GAPDH mRNA was analyzed in the same samples as a control of the amount of cDNA loaded in each lane. (**b**) HMC were transiently cotransfected with 400 ng of the human COX-2 promoter fused to the *Luciferase* reporter gene and 10 ng of TK-*Renilla* plasmid for 5 h in culture medium containing 10% FBS. Afterwards, cells were deprived of serum for 24 h and treated with TGF-β1 (1 ng/ml) for 12 h and luciferase and *Renilla* activities were determined (*$P<0.01$). (**c**) Serum-starved HMC were stimulated with TGF-β1 (1 ng/ml) in serum-free medium for 12 h. At this time point (0 h, maximum COX-2 mRNA expression), two sets of HMC were maintained in the original serum-free medium containing TGF-β1. One of them was left untreated and the other treated with the transcriptional inhibitor Act D (5 μg/ml). Fresh serum-free medium (without TGF-β1) was added to another set of cells treated with Act D. Total RNA was isolated at each time point, and COX-2 mRNA levels were analyzed by semiquantitative RT-PCR. Relative levels of COX-2 mRNA expression were determined by densitometric scanning of the bands and normalized to the GAPDH signal. Values depicted for mRNA abundance were expressed as a percentage of the control (0 h). Results are expressed as the mean ± s.e.m. of four independent experiments.

Multiple MAPK routes participate in TGF-β1-induced COX-2 expression in HMC

TGF-β1 is known to induce a number of cellular responses via ERK1/2 (p44/p42 MAPK).[23] Furthermore, activation of ERK1/2 has been shown to mediate the induction of COX-2.[24,25] Thus, our next objective was to define the role played by ERK1/2 in TGF-β1-induced COX-2 expression in HMC. For this purpose, HMC were stimulated with TGF-β1

(1 ng/ml) and ERK1/2 activation was assessed by Western blot of phospho-ERK. We also studied the effect of inhibiting the ERK1/2 pathway with the MAP-kinase kinase (MEK) inhibitors PD98059 and U0126 on TGF-β1-induced COX-2 expression and PGE_2 production. TGF-β1 increased ERK1/2 phosphorylation that reached a maximum after 15 min and then decreased progressively (Figure 4a). Total ERK1/2 remained constant throughout the duration of the experiment. Inhibition of ERK1/2 activation by the MEK inhibitors PD98059 (50 μM) or U0126 (10 μM) notably decreased the TGF-β1-dependent COX-2 protein induction in HMC (Figure 4b). Furthermore, PGE_2 production in response to TGF-β1 was markedly reduced in HMC treated with PD98059 or U0126 (Figure 4c). We also confirmed the effectiveness of PD98059 and U0126 in blocking MEK-dependent activation of ERK1/2 by TGF-β1. As our data demonstrate, both substances effectively blocked ERK1/2 phosphorylation in basal conditions and after incubation with TGF-β1 (Figure 4d). Taken together, these results indicate that the ERK1/2 MAPK pathway participates in COX-2 increase induced by TGF-β1 in HMC.

TGF-β1 is known to induce a number of cellular responses via p38 MAPK in several cell types, including MC.[26] For this reason, we have also assessed whether TGF-β1-dependent COX-2 expression was mediated by activation of p38 MAPK signalling. For this purpose, serum-starved HMC were stimulated with TGF-β1 and the level of phosphorylated p38 MAPK was measured by Western blot. TGF-β1 (1 ng/ml) promoted a modest but reproducible increase in p38 phosphorylation that reached a maximum after 15 min and then decreased (Figure 5a). Total p38 remained constant throughout the duration of the experiment (Figure 5a). To establish the role that p38 MAPK plays in TGF-β1-dependent COX-2 expression, HMC were pretreated with the inhibitor of p38 MAPK activity, SB203580 (10 μM), and then treated with TGF-β1 for 24 h. We observed that SB203580 reduced TGF-β1-induced COX-2 protein upregulation (Figure 5b). Furthermore, the level of TGF-β1-dependent PGE_2 production was also blocked with SB203580 treatment (Figure 5c).

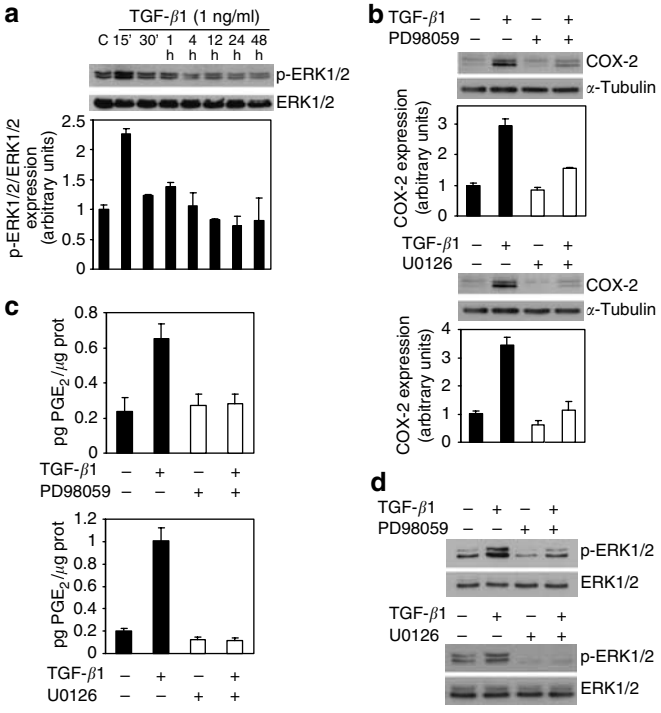

Figure 4 | ERK1/2 activation is required for TGF-β1-dependent COX-2 expression. (**a**) HMC were serum-starved for 24 h and then stimulated with TGF-β1 (1 ng/ml) for the indicated time periods. Total protein extracts were analyzed by Western blotting using specific antibodies for total and phosphorylated ERK1/2. (**b**) HMC were serum-starved for 24 h and pretreated with the MEK1/2 inhibitors PD98059 (50 μM) or U0126 (10 μM) for 30 min before stimulation with TGF-β1 (1 ng/ml) for 24 h. COX-2 protein expression levels were analyzed by Western blotting. (**c**) Cells were serum-starved for 24 h and pretreated with PD98059 (50 μM) or U0126 (10 μM) for 30 min before stimulation with TGF-β1 (1 ng/ml) for 24 h. Culture medium was collected and PGE_2 was measured by ELISA. PGE_2 levels were corrected by protein amount present in each plate. (**d**) Serum-starved HMC were pretreated with PD98059 (50 μM) or U0126 (10 μM) for 30 min before stimulation with TGF-β1 (1 ng/ml) for 15 min. Total protein extracts were analyzed by Western blotting using specific antibodies for total and phosphorylated ERK1/2. Errors bars are not shown where they are smaller than the symbol. Results are expressed as the mean $\pm$ s.e.m. of, at least, two independent experiments.

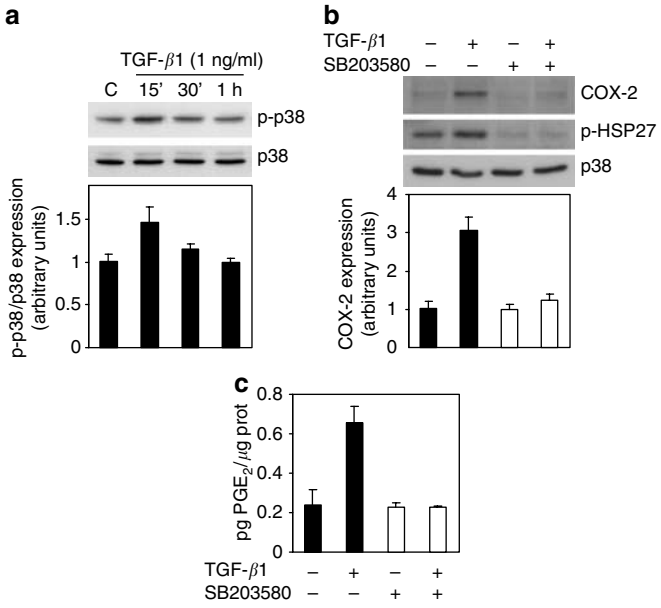

Figure 5 | TGF-β1-dependent COX-2 expression is mediated by p38 MAPK. (**a**) HMC were serum-starved for 24 h and stimulated with TGF-β1 (1 ng/ml) for the indicated time periods. Total protein extracts were obtained and analyzed for total and phosphorylated p38 using specific antibodies. (**b**) Serum-starved HMC were pretreated with the p38 MAPK inhibitor SB203580 (10 μM) for 30 min before stimulation with TGF-β1 (1 ng/ml) for 24 h (for COX-2) or 15 min (for p-HSP27 and p38). COX-2, p-HSP27, and p38 protein expression levels were analyzed by Western blotting. (**c**) Serum-starved HMC were pretreated with SB203580 (10 μM) for 30 min before stimulation with TGF-β1 (1 ng/ml) for 24 h. The culture medium was collected to measure PGE_2 by ELISA. PGE_2 levels were corrected by protein amount present in each plate. Results are expressed as the mean $\pm$ s.e.m. of, at least, two independent experiments.

We also assessed the effectiveness of SB203580 in blocking p38 MAPK activity using an indirect measure of p38 MAPK activity, the phosphorylation of HSP27.[26] HSP27 phosphorylation was stimulated by TGF-β1 treatment for 15 min. SB203580 blocked TGF-β1-stimulated HSP27 phosphorylation (Figure 5b). Taken together, these results demonstrate that the p38 MAPK pathway participates in the COX-2 increase induced by TGF-β1 in HMC.

The PI3K/Akt route participates in the regulation of TGF-β1-induced COX-2 expression

Several studies have shown the involvement of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in the regulation of COX-2 expression and PGE_2 synthesis.[27,28] As PI3K plays an important role in TGF-β1-induced collagen expression in MC,[29] our next objective was to assess the role of PI3K in TGF-β1-induced COX-2 protein expression and PGE_2 production in HMC. Our data reveal that TGF-β1 (1 ng/ml) induced a two-fold increase in Akt phosphorylation after 15 min and returned to basal values by 30 min of incubation with TGF-β1 (Figure 6a). Total Akt remained constant throughout the duration of the experiment (Figure 6a). To determine whether PI3K is involved in TGF-β1-induced COX-2 expression and PGE_2 synthesis in HMC, cells were incubated with the PI3K inhibitor LY294002. Pretreatment of HMC with LY294002 (20 μM) considerably attenuated TGF-β1-induced COX-2 expression (Figure 6b) and PGE_2 synthesis (Figure 6c). We also verified the effectiveness of LY294002 in blocking Akt activation by TGF-β1 in HMC. Our data reveal that LY294002 (20 μM) completely inhibited TGF-β1-induced Akt phosphorylation (Figure 6d). These results suggest that TGF-β1-stimulated COX-2 expression and PGE_2 synthesis are partially mediated through the activation of the PI3K/Akt pathway in HMC.

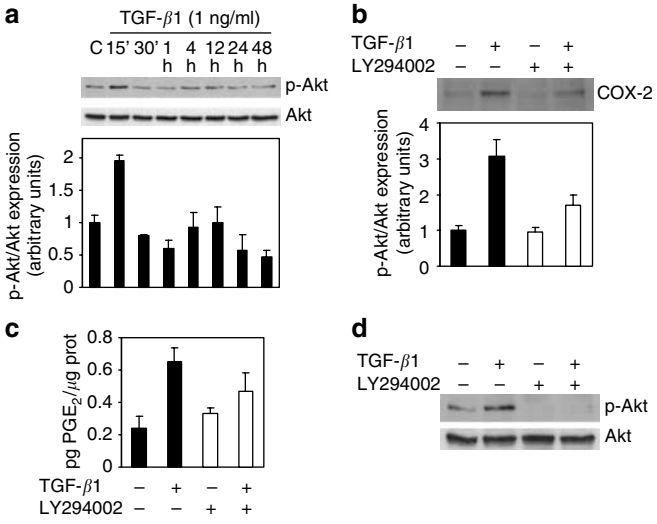

Figure 6 | PI3K/Akt pathway mediates TGF-β1-dependent COX-2 expression. (**a**) HMC were serum-starved for 24 h and then stimulated with TGF-β1 (1 ng/ml) for the indicated time periods. Total protein extracts were analyzed by Western blotting using specific antibodies for total and phosphorylated Akt. (**b**) Serum-starved HMC were pretreated with the PI3K inhibitor LY294002 (20 μM) for 30 min before stimulation with TGF-β1 (1 ng/ml) for 24 h. COX-2 protein expression levels were analyzed by Western blotting. (**c**) Serum-starved HMC were pretreated with LY294002 (20 μM) for 30 min before stimulation with TGF-β1 (1 ng/ml) for 24 h. The culture medium was collected to measure PGE_2 by ELISA. PGE_2 levels were corrected by protein amount present in each plate. (**d**) Serum-starved HMC were pretreated with LY294002 (20 μM) for 30 min before stimulation with TGF-β1 (1 ng/ml) for 15 min. Total protein extracts were analyzed by Western blotting using specific antibodies against total and phosphorylated Akt. Results are expressed as the mean ± s.e.m. of, at least, two independent experiments.

TGF-β1-induced proliferation and collagen I synthesis are independent of COX-2 activation

Our data reveal that TGF-β1 (1 ng/ml) inhibits proliferation of HMC growing in 10% fetal calf serum after 5 days of treatment (Figure 7a). The specific COX-2 inhibitor NS-398 also inhibited basal HMC proliferation, thus indicating that COX-2 induces proliferation of HMC in culture. To investigate the possible involvement of COX-2 in TGF-β1-induced inhibition of proliferation, we added NS-398 to cultured HMC 1 h before the addition of TGF-β1. The number of cells was the same as in TGF-β1 treatment, indicating that the TGF-β1-induced proliferation inhibition is independent of COX-2 activation.

Our data also reveal that TGF-β1 (1 ng/ml) increases collagen I mRNA and protein expression in HMC (Figure 7c and d). To study the role of COX-2 in TGF-β1-induced collagen I expression, we inhibited selectively COX-2 by adding NS-398 1 h before TGF-β1 treatment. Neither collagen I mRNA nor protein expression was modified by inhibition of COX-2 activity, suggesting that TGF-β1-induced collagen expression is independent of COX-2 activation.

None of the pharmacological inhibitors used – NS-398, PD98059, U0126, SB203580, and LY294002 – had toxic effect on the HMC at the concentration used in our cell culture experiments.

DISCUSSION

In this paper, we describe, for the first time, the effect of TGF-β1 on COX-2 expression in primary cultured HMC. We show that TGF-β1 upregulates COX-2 expression and PGE_2 production, and identify several signalling routes that are involved in these actions of TGF-β1.

COX-2 is an inducible enzyme involved in chronic inflammation.[30] Several studies have highlighted an upregulation of COX-2 expression in proliferative glomerulonephritis.[31,32] In this study, we demonstrate that TGF-β1 activates COX-2 expression and PGE_2 production, whereas TGF-β1 does not affect COX-1 expression in primary cultured HMC. This effect is specific for TGF-β1 because the sTβRII inhibits the effects of TGF-β1 on COX-2 expression and PGE_2 production. Furthermore, the PGE_2 production in response to TGF-β1 is dependent on COX-2 activity, as pretreatment with the selective COX-2 inhibitor NS-398 blocked TGF-β1-induced PGE_2 production.

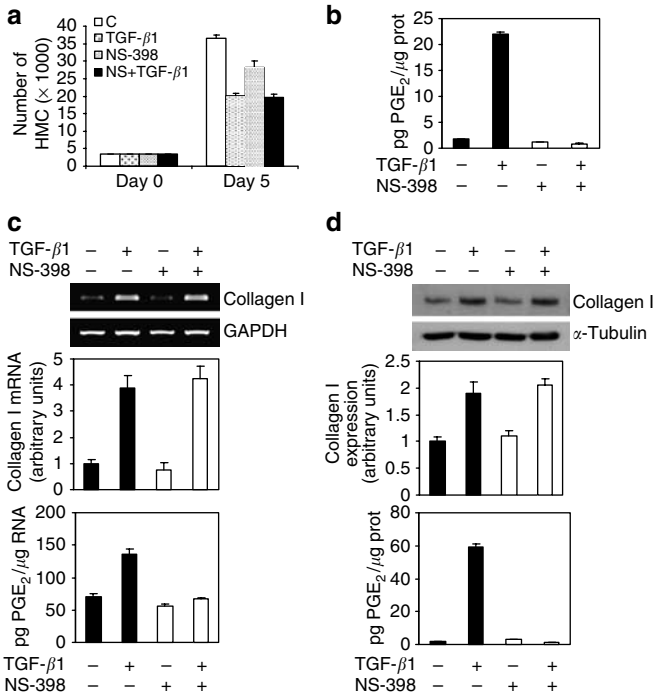

Figure 7 | COX-2 mediates proliferation but not collagen expression induced by TGF-β1 in HMC. (**a**) HMC were plated in 24-well plates to a density of 4000 per well. The following day, HMC were stimulated with TGF-β1 (1 ng/ml), the specific COX-2 inhibitor NS-398 (10 μM), or a combination of both (adding NS-398 1 h before TGF-β1) in the presence of 10% FBS. Cell proliferation was analyzed at days 0 (day of treatment) and 5 by an MTT assay. Results are expressed as number of HMC using a curve correlating number of HMC and absorbance at 595 nm, and represent the mean ± s.e.m. of three experiments performed in duplicate. (**b**) At the time, HMC were plated in 60 mm dishes and treated for 5 days with the previous agonist. The culture medium was collected and PGE_2 concentration was measured by ELISA. (**c**) HMC were treated with TGF-β1 (1 ng/ml), NS-398 (10 μM), or a combination of both (adding NS-398 1 h before TGF-β1) for 8 h in the presence of 10% FBS. Total RNA was then isolated and RT-PCR of collagen I (α1) was performed. PGE_2 concentration was also measured in the culture medium. (**d**) HMC were treated with TGF-β1 (1 ng/ml), NS-398 (10 μM), or a combination of both for 48 h in the presence of 10% FBS. Total protein extracts were analyzed by Western blotting using a specific antibody for collagen I. PGE_2 concentration was also measured in the culture medium.

Although TGF-β1 is known to induce COX-2 expression in various cell types,[15–17] this is the first time that it has been demonstrated in HMC.

The regulation of COX-2 expression has been shown to occur at both transcriptional and post-transcriptional levels. The stability of COX-2 mRNA is an important regulatory mechanism of COX-2 expression.[33] In the present study, we demonstrate that administration of TGF-β1 increased the COX-2 promoter activity and the amount of mRNA for this gene in HMC. In addition, TGF-β1-induced COX-2 mRNA expression was inhibited by Act D. All these data suggest that *de novo* transcription is required in this process.

It is well recognized that the Smad family of proteins are key intracellular mediators of TGF-β1 signalling.[34] Although much effort has been made to identify the signalling pathways triggered by TGF-β1, the signal transduction mechanisms by which TGF-β1 induces COX-2 protein expression and PG production are still unclear. Several recent reports indicate that an important group of protein kinases may be involved in these processes. This signalling mechanism is believed to control cellular physiological and pathophysiological processes including cell growth, differentiation, and stress responses.[35] Recent work suggests that the MAPK pathway is also involved in regulating PG biosynthesis.[36–38] Thus, we have assessed the role of MAPK activation in TGF-β1 induced COX-2 expression and increased PGE_2 synthesis in HMC. In this way, another major result of this study is that TGF-β1 induces ERK1/2 and p38 MAPK phosphorylation in a time-dependent manner. These data are in agreement with those of Chin *et al.*,[39] reporting that TGF-β1 is able to activate the ERK1/2 and the p38 MAPK, but not JNK, in rat glomerular MC. In addition, our data demonstrate that pharmacological inhibition of ERK1/2 and p38 MAPK inhibits TGF-β1-mediated COX-2 upregulation and PGE_2 production. Some findings indicate that SB203580 and PD98059 can directly inhibit thromboxane synthase, and COX-1 and -2, and thus some of the effects of these inhibitors on PGE_2 production could be due to COX inhibition and alteration in arachidonic acid metabolism.[40] However, it should be noted that our results demonstrate that these inhibitors also decreased the amount of COX-2 detected by Western blot, thus demonstrating that they are effective in inhibiting COX-2 synthesis. All these data suggest that in primary cultures of HMC, activation of ERK1/2 and p38 MAPK mediates TGF-β1-induced COX-2 expression and increased PGE_2 production.

TGF-β1 has been reported to activate or inhibit PI3K signalling depending on the cell type and the stimulus.[41–45] Our results also provide evidence that Akt/PKB activation plays an important role in TGF-β1-induced expression of COX-2. The PI3K downstream target Akt is rapidly activated by TGF-β1 in HMC. Furthermore, we show that inhibition of PI3K with LY294002 partially blocks TGF-β1-stimulated COX-2 expression. Similarly, LY294002 reduces the increase in PGE_2 production induced by TGF-β1. Taken together, the above results suggest that PI3K activation participates in TGF-β1-mediated full induction of both COX-2 protein expression and PGE_2 production in human renal MC.

TGF-β is an important cytokine in glomerular disease. Its major role may be to mediate extracellular matrix deposition; however, TGF-β may also have other important functions in the glomerulus, including the regulation of cell proliferation, hypertrophy, and survival, as well as modulation of the local and systemic immune response. As expected, in our study, TGF-β induced collagen synthesis and accumulation and reduced proliferation in HMC. However, neither collagen type I mRNA, nor net collagen production, nor cell proliferation was altered by co-treatment with the COX-2 inhibitor NS398. These results suggest that COX-2-derived prostanoids do not directly interfere with TGF-β1-induced

inhibition of MC proliferation or extracellular matrix synthesis '*in vitro*', although probably could play a role in glomerular hemodynamics or glomerular inflammation.

In conclusion, our results demonstrate for the first time that in primary cultures of HMC, TGF-β1 induces the expression of COX-2 and increases the production of PGE_2. These effects seem to be mediated, at least partially, by the activation of ERK1/2, p38 MAPK, and PI3K pathways. These biochemical events may be implicated in normal renal physiology as well as in the pathogenesis of glomerular diseases. These results could be useful in elucidating the molecular mechanisms underlying the regulation of COX-2 in MC and may open up specific strategies for the treatment of renal glomerular inflammatory diseases that specifically target the pathways involved in COX-2 activation by TGF-β1.

MATERIALS AND METHODS

Materials

TGF-β1 was from R&D Systems (Minneapolis, MN, USA) and Act D was from Sigma (Madrid, Spain). NS-398, PD98059, U0126, SB203580, LY294002, and anti-α-tubulin antibody were from Calbiochem (Darmstadt, Germany). The sTβRII[46] was provided by Calvin Vary (Center for Molecular Medicine, Maine Medical Center Research Institute, Scarborough, ME, USA). Antibodies to COX-1, COX-2, p-ERK1/2, ERK2, Akt, and p38 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p-Akt antibody was from BD Biosciences (Madrid, Spain), anti-collagen I antibody was from Chemicon (Temecula, CA, USA), and anti-p-p38 and -p-HSP27 were form Cell Signalling Technology (Beverly, MA, USA).

Cell culture

Primary cultures of HMC were obtained from glomeruli prepared from the cortex of the intact pole of kidneys removed for circumscribed tumor, as described previously.[47] All kidney tissue was distant from the neoplasm and macroscopically normal. Histological examination of these samples revealed no renal pathology. Cells were maintained in RPMI 1640 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS) (GibcoBRL Life Technologies, Paisley, Scotland), penicillin (50 U/ml), streptomycin (50 μg/ml), sodium selenite (5 ng/ml), and insulin and transferrin (5 μg/ml each) at 37°C in the presence of 5% CO_2. Cells were confirmed as mesangial by standard morphological and functional criteria.[48] Experiments were performed from passages 4 to 8. Immortalized HMC (clone 18) were grown in Dulbecco's modified Eagle's medium in the presence of 10% FBS, penicillin (100 U/ml), and streptomycin (100 U/ml) as described.[49]

Plasmids, transfection, and luciferase reporter assay

To determine the activity of the COX-2 promoter, 400 ng of the human COX-2 promoter cloned in the reporter plasmid pXP2 (P2-1900)[22] (a gift from Miguel A Iñiguez, Centro de Biología Molecular 'Severo Ochoa', CSIC, Madrid, Spain) and 10 ng of pRL-TK vector containing the *Renilla* luciferase gene (Promega, Madison, WI, USA), used as a control of transfection efficiency, were transiently cotransfected in immortalized HMC. The day before transfection, cells were plated into 24-well plates. Cells were transfected in culture medium containing 10% FBS for 5 h using jetPEI transfection reagent (Polyplus Transfection, Illkirch, France) according to the manufacturer's instructions. Cells were grown in FBS-free medium for 24 h and treated with TGF-β1 (1 ng/ml) for 12 h. Luciferase and *Renilla* activities were measured using a dual-reporter assay kit (Promega, Madison, WI, USA).

Western blot analyses

Western blot analysis was performed as previously described.[50] Cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 10 mM ethylenediaminetetraacetic acid, 10% glycerol, 1% Nonidet P-40, aprotinin, pepstatin, and leupeptin at 1 μg/ml each, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and were blotted onto polyvinylidine difluoride membranes. After incubation with horseradish peroxidase-conjugated secondary antibodies, bands were visualized by a luminol-based detection system with *p*-iodophenol enhancement. Anti-α-tubulin antibody was used to confirm equal loading of protein in each lane. Protein expression was quantified by densitometry using Scion Image software (Scion, Frederick, MD, USA).

RT-PCR analyses

Total RNA was isolated using TriReagent (Molecular Research Center Inc., Cincinnati, OH, USA) and chloroform extraction according to the manufacturer's instructions. A 2 μg portion of total RNA served as template for first-strand cDNA synthesis in a reaction using oligo(dT) primers and M-MLV reverse transcriptase (Promega, Madison, WI, USA). A 0.5 μg portion of cDNA was used in a standard 50 μl PCR mixture with 2 ng/μl of each primer and 2 U of FastStart *Taq* DNA polymerase (Roche, Penzberg, Germany). PCR products were obtained after 35 (for COX-2), 24 (for collagen I (α1)), or 28 (for GAPDH) cycles of amplification with an annealing temperature of 58°C (for COX-2), 52°C (for collagen I (α1)), or 60°C (for GAPDH). Primer sequences were as follows: for *COX-2*,[25] forward: 5′-TTCAAATGAGATTGTGGGAAAAT-3′; reverse: 5′-AGA TCATCTCTGCCTGAGTATCTT-3′ (amplifies a fragment of 305 bp); wfor *collagen I* (*α1*), forward: 5′-TGTTGCTGAAAGACTACCTCGT-3′; reverse: 5′-CCTCCCATGTTAAATAGCACCT-3′ (amplifies a fragment of 303 bp); for *GAPDH*, forward: 5′-TGAAGGTCGGTGTGAACG GATTTGGC-3′; reverse: 5′-CATGTAGGCCATGAGGTCCACCAC-3′ (amplifies a fragment of 900 bp). PCR products were analyzed by 1% agarose gel electrophoresis containing ethidium bromide and quantitated digitally using Scion Image software.

PGE_2 measurements

HMC plated on 60 mm culture plates were grown in FBS-free medium for 24 h and treated with TGF-β1 (1 ng/ml) during time periods referred in each experiment. PGE_2 concentration in the culture medium was measured by enzyme-linked immunosorbent assay (ELISA) using a PGE_2 High Sensitivity Immunoassay kit (R&D Systems, Minneapolis, MN, USA). PGE_2 concentration was corrected by the protein content in each culture plate. The protein content was determined by a commercially available variant of the Lowry method (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as standard.

Cell proliferation measurements

HMC were plated on 24-well plates to a density of 4000 per well and allowed to attach overnight. Cells were then treated with 1 ng/ml TGF-β1, 10 μM NS-398, or a combination of both in the presence

of 10% FBS. Cell proliferation was analyzed at days 0 and 5 of treatment by a 3-[4,5]dimethylthiazol-2,5-diphenyltetrazolium bromide-based assay (Roche) as per the manufacturer's recommendations. The absorbance of the samples was then measured at 595 nm, and the results transformed in cell number with a curve that correlated absorbance and number of HMC.

Cytotoxicity assay

HMC were treated with and without pharmacological inhibitors: 10 μM NS-398, 50 μM PD98059, 10 μM U0126, 10 μM SB203580, and 20 μM LY 294002 and cytotoxicity was assessed by the Toxilight BioAssay Kit (Cambrex, Verviers, Belgium), a commercial kit that quantitatively measures the release of adenylate kinase from damaged cells.

Statistical analysis

All the values are presented as the mean showing the s.e.m. Statistical analysis for luciferase reporter assay was performed with the Student's *t*-test using SPSS 12.0 software (SPSS, Chicago, IL, USA).

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