Sphingosylphosphorylcholine acts in an anti-inflammatory manner in renal mesangial cells by reducing interleukin-1β-induced prostaglandin E_2 formation

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Abstract Sphingosylphosphorylcholine (SPC) is a bioactive lipid that binds to G protein-coupled-receptors and activates various signaling cascades. Here, we show that in renal mesangial cells, SPC not only activates various protein kinase cascades but also activates Smad proteins, which are classical members of the transforming growth factor-β (TGFβ) signaling pathway. Consequently, SPC is able to mimic TGFβ-mediated cell responses, such as an anti-inflammatory and a profibrotic response. Interleukin-1β-stimulated prostaglandin E_2 formation is dose-dependently suppressed by SPC, which is paralleled by reduced secretory phospholipase A_2 ($sPLA_2$) protein expression and activity. This effect is due to a reduction of $sPLA_2$ mRNA expression caused by inhibited $sPLA_2$ promoter activity. Furthermore, SPC upregulates the profibrotic connective tissue growth factor (CTGF) protein and mRNA expression. Blocking TGFβ signaling by a TGFβ receptor kinase inhibitor causes an inhibition of SPC-stimulated Smad activation and reverses both the negative effect of SPC on $sPLA_2$ expression and the positive effect on CTGF expression. In summary, our data show that SPC, by mimicking TGFβ, leads to a suppression of proinflammatory mediator production and stimulates a profibrotic cell response that is often the end point of an anti-inflammatory reaction. Thus, targeting SPC receptors may represent a novel therapeutic strategy to cope with inflammatory diseases.—Xin, C., S. Ren, W. Eberhardt, J. Pfeilschifter, and A. Huwiler. **Sphingosylphosphorylcholine acts in an anti-inflammatory manner in renal mesangial cells by reducing interleukin-1β-induced prostaglandin E_2 formation. *J. Lipid Res.* 2007.** 48: **1985–1996.**

Supplementary key words transforming growth factor-β_2 • Smad • secretory phospholipase A_2 • connective tissue growth factor

Sphingosylphosphorylcholine (SPC) is a lysosphingolipid showing structural similarities to sphingosine 1-phosphate (S1P) and lysophosphatidylcholine (LPC). Similar to S1P and LPC, SPC acts by binding to and activating cell surface receptors and thereby triggers various cell responses, such as vasoconstriction or vasodilation, angiogenesis, stress fiber formation, and cytoskeletal rearrangements (1, 2).

High-affinity SPC receptors have been reported, including G protein-coupled receptor 4 (GPR4) (3), GPR12 (4), GPR68 (OGR1) (5), and G2A (6), whereas low-affinity SPC binding receptors include the S1P receptors (reviewed in Refs. 1, 2). However, the identity of these receptors as high-affinity SPC receptors remains controversial and unclear, as several of these first reports were retracted. Recently, G2A and GPR68/OGR1 were shown to be activated by decreasing the pH rather than by SPC (7). Furthermore, GPR12 was reported as unresponsive to SPC (8), and SPC binding to GPR4 could not be confirmed by Bektas et al. (9). Considering these latter negative reports, it is clear that the high-affinity SPC receptors responsible for SPC-triggered signal transduction and SPC-induced cell responses still need to be identified.

Physiologically, SPC and S1P have both been shown to exert renal effects in vivo. Intravenous bolus injection of S1P, but not SPC, dose-dependently reduced renal blood flow, whereas both sphingolipids exerted a similar effect on the tubular system to enhance diuresis and natriuresis (10). The reduced renal blood flow by S1P is attributable to a contraction of intrarenal resistance vessels (11, 12), whereas the diuretic effects of S1P and SPC are probably based on an alteration of transport processes in the distal part of the nephron (11, 12).

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Abbreviations: COX-2, cyclooxygenase-2; CTGF, connective tissue growth factor; GPR4, G protein-coupled receptor 4; IL-1β, interleukin-1β; LPC, lysophosphatidylcholine; MAPK, mitogen-activated protein kinase; PGE_2, prostaglandin E_2; PKB, protein kinase B; PLA_2, phospholipase A_2; S1P, sphingosine 1-phosphate; SBE, Smad binding element; siRNA, small interfering RNA; SM, sphingomyelin; SPC, sphingosylphosphorylcholine; $sPLA_2$, secretory phospholipase A_2; TGFβ, transforming growth factor-β; TIE, transforming growth factor-β inhibitory element; TKI, transforming growth factor-β receptor type I kinase inhibitor.

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One of the early effects triggered by SPC and S1P in various cell culture systems is an intracellular Ca^{2+} mobilization. In mesangial cells, it was previously shown that SPC- and S1P-stimulated Ca^{2+} mobilization occur via different mechanisms (13). Whereas S1P acted exclusively through PLC activation and inositol 1,4,5-trisphosphate generation to mobilize Ca^{2+} from intracellular stores, SPC additionally also stimulated Ca^{2+} influx (13). Again, this highlights the fact that SPC and S1P trigger divergent signaling cascades.

Renal mesangial cells are involved in the regulation of the glomerular filtration rate as well as in the preservation of the structural integrity of the glomerulus. Furthermore, they play a central role in most pathological processes of the renal glomerulus (14–16). Several proinflammatory functions of mesangial cells have been established, including: *i*) increased extracellular matrix production, *ii*) increased inflammatory mediator production, and *iii*) increased mesangial cell proliferation, which are all hallmarks of many forms of glomerulonephritis (14, 15). However, the detailed mechanisms underlying these cell responses are still not completely understood. A particularly important factor in the mechanism of matrix accumulation is transforming growth factor-β (TGFβ), whose production is highly induced in many fibrotic diseases, including atherosclerosis and fibrosis of the kidney, liver, and lung (17–19).

In this study, we show that SPC is able to act on mesangial cells to rapidly activate various signaling cascades, including the mitogen- and stress-activated protein kinases, protein kinase B (PKB), and the Smad proteins, which are members of the TGFβ signaling device. This cross-activation of the TGFβ/Smad signaling cascade leads on the one hand to an inhibition of interleukin-1β (IL-1β)-induced group IIA secretory phospholipase A_2 ($sPLA_2$) protein expression, mRNA expression, and promoter activity and on the other hand to an activation of gene transcription of the profibrotic connective tissue growth factor (CTGF).

MATERIALS AND METHODS

Chemicals

S1P and SPC were obtained from Biotrend (Köln, Germany); IL-1β was obtained from Cell Concept (Umkirch, Germany); $TGF\beta_2$ was from R&D Systems (Wiesbaden, Germany); and the TGFβ receptor type I kinase inhibitor was from Merck Biosciences (Schwalbach, Germany). The Rediprime II random prime labeling system, Nick columns, prostaglandin E_2 (PGE_2) ELISA, [α-^{32}P]dCTP, hyperfilm MP, and horseradish-coupled secondary antibodies were from Amersham Pharmacia Biotech (Freiburg, Germany). All phospho-specific antibodies were from Cell Signaling (Frankfurt am Main, Germany); monoclonal rat $sPLA_2$ antibody was kindly provided by Henk van den Bosch (20). The total Smad-3 (FL-425) antibody (sc-8332), Smad-4 (B-8) antibody (sc-7966), CTGF (L-20) antibody (sc-14939), and cyclooxygenase-2 (COX-2; M-19) antibody (sc-1747) were from Santa Cruz Biotechnology (Heidelberg, Germany); Trizol and all cell culture nutrients were from Gibco Invitrogen (Karlsruhe, Germany).

Cell culture

Rat renal mesangial cells were cultivated and characterized as described previously (21). Passages 9–22 were used for the experiments in this study.

Cell stimulation and Western blot analysis

Confluent mesangial cells in 60 mm diameter dishes were rendered quiescent by incubation for 20 h in DMEM containing 0.1 mg/ml fatty acid-free BSA in the same medium as indicated. Thereafter, the medium was withdrawn and the cells were washed once with ice-cold PBS solution. Cells were scraped into ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 40 mM β-glycerophosphate, 50 mM sodium fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM pepstatin A, and 1 mM PMSF) and homogenized by 10 passes through a 26 gauge needle fitted to a 1 ml syringe. Samples were centrifuged for 10 min at 16,000 *g*, and the supernatant was taken for protein determination. Cell extracts containing 50 μg of protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane followed by immunostaining, as described previously in detail (22). Antibodies were diluted in blocking buffer as indicated in the figure legends. Bands were detected by the ECL method as recommended by the manufacturer.

Nuclear fractionation of proteins

Stimulated cells were scraped into ice-cold buffer containing 0.5 mM EDTA in PBS and centrifuged for 1 min at 2,300 *g* at 4°C. The pellet was resuspended in buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 10 μg/ml leupeptin, 1 μM pepstatin A, 10 mM DTT, and 2 mM PMSF and incubated for 15 min at 4°C. After adding 10% (v/v) Nonidet P40, the cell lysate was centrifuged for 1 min at 16,000 *g*. The pellet was further processed by adding buffer containing 20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 10 μg/ml leupeptin, 1 μM pepstatin A, 10 mM DTT, and 2 mM PMSF, resuspended by vigorous vortexing for 20 min at 4°C, and centrifuged for 20 min at 16,000 rpm. The supernatant contained nuclear protein and was taken for protein determination.

$sPLA_2$ secretion and immunostaining

Equal volumes of supernatants derived from the same number of cells were taken for protein precipitation using 10% (w/v) trichloroacetic acid. Precipitated proteins were separated by SDS-PAGE (13% acrylamide gel), transferred to nitrocellulose membranes, and immunostained using a monoclonal antibody against rat group IIA $sPLA_2$ at a dilution of 1:60, as described previously (20).

$sPLA_2$ activity assay

Equal volumes of supernatants were taken for an in vitro assay using [^{14}C]oleic acid-labeled *Escherichia coli* as a substrate (23) in a total volume of 0.2 ml including 20 mM Tris-HCl, pH 8.5, and 10 mM $CaCl_2$. Samples were incubated for 60 min at 37°C and stopped by the addition of 2.5 ml of Dole reagent [isopropyl alcohol-heptane-2 N H_2SO_4 (40:10:1)]. Liberated ^{14}C-labeled fatty acids were extracted by adding 1.5 ml of heptane and 1 ml of water followed by vigorous vortexing. The heptane phase was loaded onto a silica gel column, and ^{14}C-labeled free fatty acids were eluted with diethylether and counted in a β-counter.

PGE_2 determination

Equal volumes of supernatants were subjected to a PGE_2 ELISA according to the manufacturer's instructions.

Northern blot analysis

Total RNA was isolated using guanidinium isothiocyanate solution. Twenty micrograms of RNA was separated by electrophoresis on a 1% agarose-formaldehyde gel. RNA was transferred to a nylon membrane and cross-linked by ultraviolet light. Blots were hybridized with a 416 bp RT-PCR product of rat IIA $sPLA_2$ (forward primer, GGT CCT CCT GTT GCT AGC AG; reverse primer, CTT TGC AAA ACT TGT TGG GG). The probe was labeled with [α-^{32}P]dCTP using the Multiprime DNA Labeling System (Amersham Pharmacia Biotech). Hybridization was carried out at 42°C for 20 h, and the washed membranes were exposed and analyzed on an Imaging system (Fuji). To correct for variations in RNA amounts, blots were finally rehybridized with a ^{32}P-labeled GAPDH cDNA probe.

Determination of nitric oxide formation

Quiescent mesangial cells on 24-well plates were stimulated as indicated, and the supernatant was taken for nitrite determination by mixing 50 μl of the supernatant with 37.5 μl of Griess reagent. Absorbance at 450 nm was measured in a 96-well spectrophotometer (Tecan). For quantification, a standard curve in the range of 1–100 μM sodium nitrite was used.

Small interfering RNA transfections

Gene silencing was performed using sequence-specific small interfering RNA (siRNA) reagents: rat Smad-4 (AAUACACCGACAAGCAAUGACdTdT and GUCAUUGCUUGUCGGUGUAUUdTdT) and rat TGFβ RII (AAAGUCGGUUAACAGCGAUCUdTdT and AGAUCGCUGUUAACCGACUUUdTdT). Mesangial cells were transfected at 30–50% confluence with 200 nM of the 21 nucleotide duplexes using Oligofectamine, as recommended by the manufacturer (Dharmacon Research, Inc., Boulder, CO). After 48–72 h, cells were stimulated as indicated in the figure legends. The silencing efficiency was verified by Western blot analyses using specific antibodies.

$sPLA_2$ promoter studies

A 2.67 kb promoter fragment of rat IIA $sPLA_2$ was cloned from rat genomic DNA by PCR using the following primers: forward, GCG CCG ACG CGT GAA AAT CCC TGA CTT GAT TC; reverse, GCG CCG CTC GAG GTT TTT CCT GTA CTC CCA ATG, according to a previous report (24) and fused into the luciferase reporter gene-containing vector pGL3. Mesangial cells were cultured on 12-well plates and transfected with 400 ng of plasmid DNA plus 100 ng of Renilla luciferase DNA per well using the Effectene transfection reagent according to the manufacturer's recommendations. Values for IIA $sPLA_2$ promoter activity were calculated from the ratio of firefly to Renilla luciferase activities and expressed as relative luciferase units.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons (GraphPad InStat version 3.00 for Windows NT; GraphPad Software, San Diego, CA).

RESULTS

SPC activates the various mitogen-activated protein kinases and the Smad signaling cascades in renal mesangial cells

Stimulation of quiescent renal mesangial cells with SPC led to a very rapid phosphorylation and thereby activation of the classical mitogen-activated protein kinases (MAPKs) p42- and p44-MAPK (**Fig. 1A**). Maximal activation was already seen after 5 min of stimulation, which thereafter declined rapidly. At 60 min, a second increase of p42- and p44-MAPK phosphorylation occurred (Fig. 1A). Because a delayed and sustained phase of MAPK activation has been suggested as a prerequisite for cell proliferation (22, 25), this increase at 60 min may be an indication of the proliferative nature of SPC reported previously by others (26). The stress-activated protein kinases p38-MAPK and stress-activated protein kinase/c-Jun N-terminal kinase as well as PKB/Akt were all phosphorylated with similar kinetics

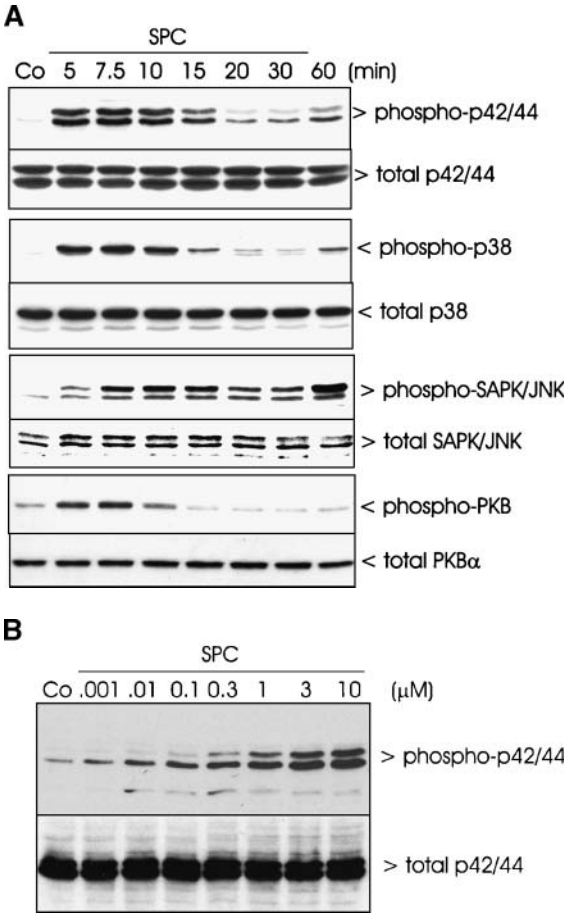

Fig. 1. Time- and concentration-dependent effects of sphingosylphosphorylcholine (SPC) on the activation of the p42/p44-mitogen-activated protein kinases (MAPKs), p38-MAPK, stress-activated protein kinase/c-Jun N-terminal kinase, and protein kinase B (PKB) cascades in renal mesangial cells. A: Quiescent rat renal mesangial cells were stimulated with either vehicle [control (Co); 5 min] or SPC (3 μM) for the indicated time periods. B: Cells were stimulated for 5 min with the indicated concentrations of SPC. Thereafter, protein extracts containing equal amounts of proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to Western blot analysis using specific antibodies against phospho-p42/p44-MAPKs (1:1,000), total p42/p44 (1:1,000), phospho-p38-MAPK (1:1,000), total p38-MAPK (1:1,000), phospho-SAPK/JNK (1:1,000), total p45-SAPK (1:1,000), phospho-Ser^{473}-PKB (1:1,000), and total PKB (1:1,000). Bands were visualized by the ECL method according to the manufacturer's instructions. Data are representative of two to three independent experiments giving similar results.

(Fig. 1A), whereas the total protein levels of these enzymes did not change during the stimulation periods (Fig. 1A). The activation of the MAPKs by SPC also occurred in a dose-dependent manner; it showed a clear increase of phosphorylation after 5 min at 10 nM and reached maximal levels at 10 μM SPC (Fig. 1B).

Furthermore, we found that the Smad signaling pathway, which is classically activated by TGFβ, was also activated in a time-dependent manner by SPC. Notably, the maximal effect of SPC on Smad-1 (**Fig. 2A**) and Smad-2 (Fig. 2B) phosphorylation was seen at 15–20 min of stimulation, which occurred later compared with the very rapid activation of p42/p44-MAPKs, p38-MAPK, stress-activated protein kinase/N-terminal c-Jun kinase, and PKB. Smad-3 was maximally phosphorylated at 5–10 min (Fig. 2C). Because the total Smad-3 antibody (FL-425) is know to cross-react with Smad-2 and to a lesser extent also with Smad-1, -5, and -8, several bands were seen (Fig. 2C, inset). None of these bands was altered upon stimulation. Moreover, the Smad-1, -2, and -3 phosphorylations at 15 min of stimulation increased in a concentration-dependent manner (Fig. 2D–F). To determine whether the increased phos-

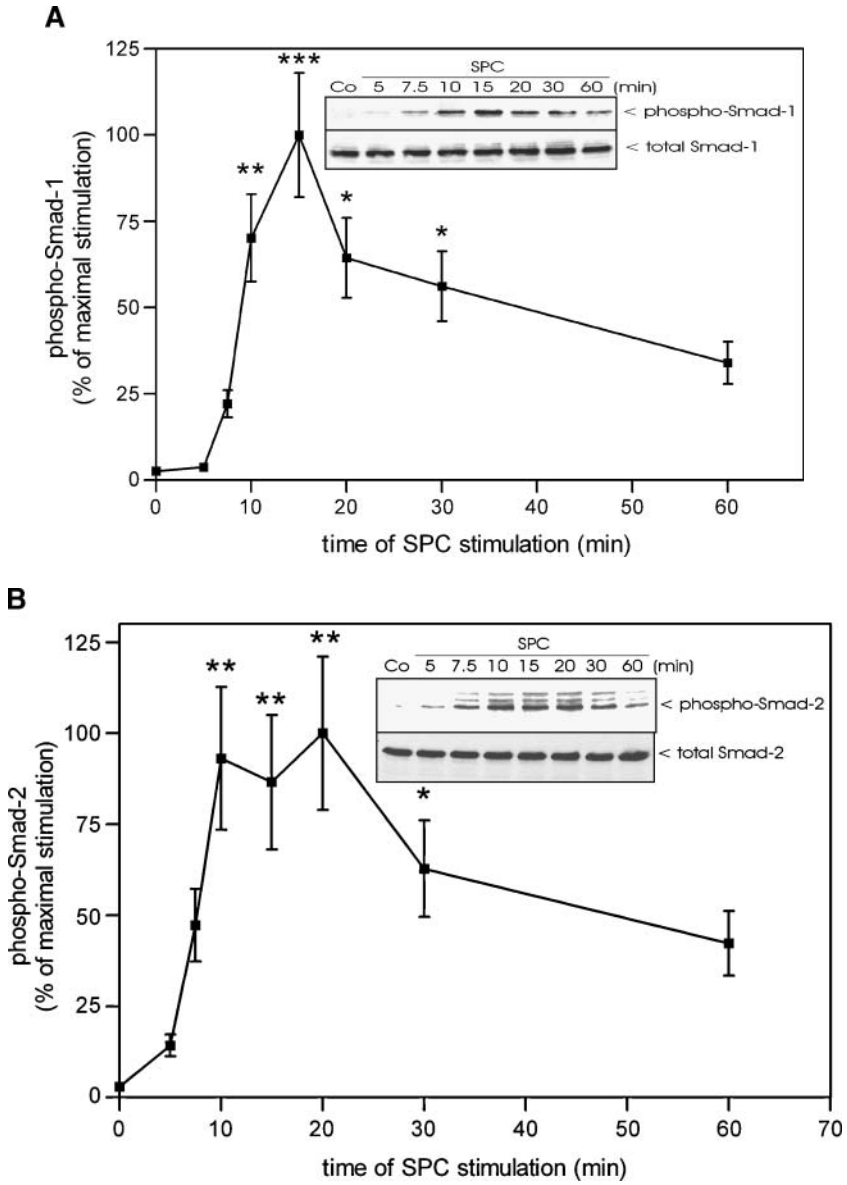

Fig. 2. Time- and concentration-dependent effects of SPC on the activation of Smad proteins in renal mesangial cells. A–C: Quiescent mesangial cells were stimulated with either vehicle [control (Co); 15 min] or SPC (3 μM) for the indicated time periods. D–F: Cells were stimulated for 15 min with the indicated concentrations of SPC. Thereafter, total cell lysates containing equal amounts of proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to Western blot analysis using phospho-specific and total antibodies against Smad-1 (inset in A), Smad-2 (inset in B), or Smad-3 (inset in C) (all at dilutions of 1:1,000). Bands were visualized by the ECL method according to the manufacturer's instructions, and bands corresponding to the Smad proteins were densitometrically evaluated. Results are expressed as percentages of maximal SPC stimulation and are means ± SD (n = 2–3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, considered statistically significant compared with vehicle-stimulated control values.

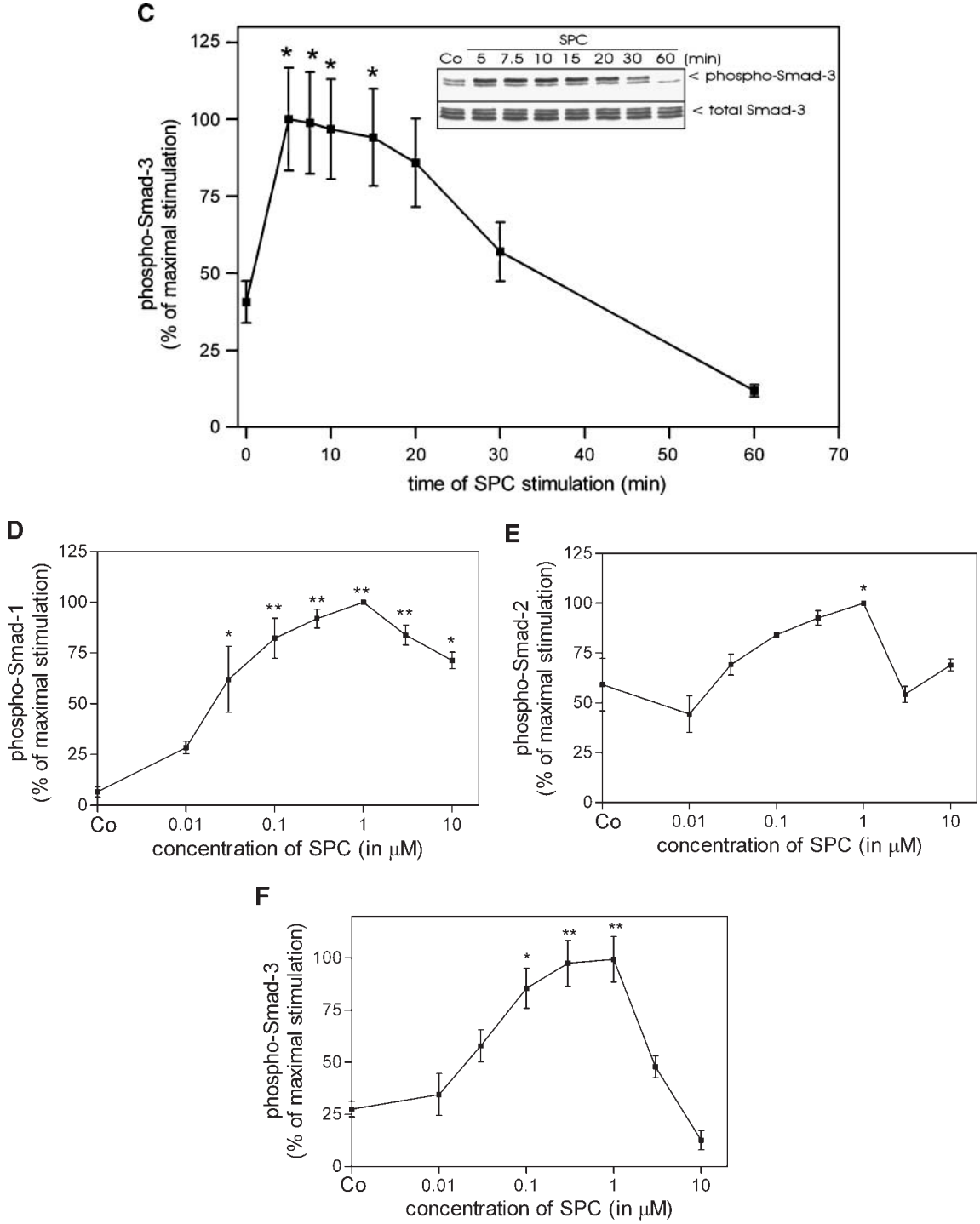

Fig. 2. Continued.

phorylation of Smads is associated with an activation of the cascade, translocation of the coregulatory Smad-4 to the nucleus was investigated upon fractionation of cell lysates. As seen in **Fig. 3**, unstimulated cells contained only a minor amount of Smad-4 in the nucleus. However, upon $TGF\beta_2$ stimulation, an accumulation of Smad-4 in the nucleus was seen that remained for up to 3 h of stimulation. In addition, SPC-stimulated cells also showed increased staining of Smad-4 in the nucleus, although this effect was more transient and less pronounced than that seen for $TGF\beta_2$. This cross-activation of the TGFβ/Smad cascade by SPC critically depended on the TGFβ receptor complex, because a recently developed TGFβ receptor type I kinase inhibitor (27) dose-dependently reduced SPC-triggered Smad phosphorylation (**Fig. 4A**). In addition, the Smad-1 phosphorylation was also abolished when depleting the TGFβ receptor type II by siRNA transfection of the cells (Fig. 4B).

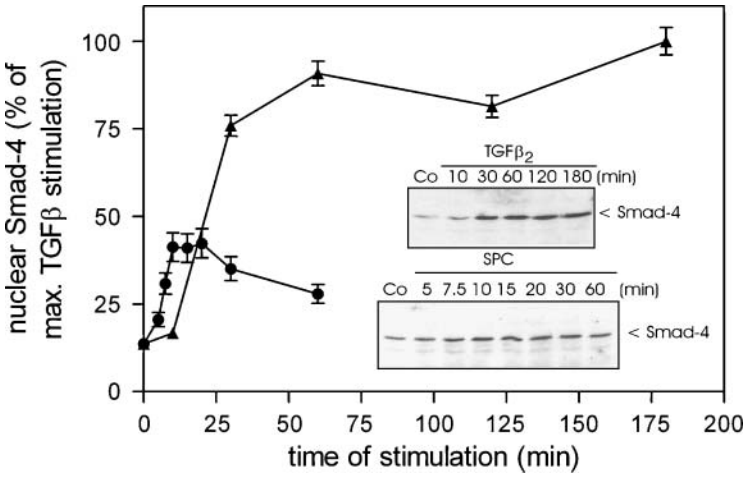

Fig. 3. Effects of transforming growth factor-β ($TGFβ_2$) and SPC on Smad-4 translocation to the nucleus in renal mesangial cells. Quiescent cells were stimulated with vehicle [control (Co); 15 min], $TGFβ_2$ (10 ng/ml; upper inset), or SPC (3 μM; lower inset) for the indicated time periods. Thereafter, nuclear extracts were prepared as described in Materials and Methods, and proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to Western blot analysis using a specific antibody against Smad-4 (1:1,000). Bands were visualized by the ECL method according to the manufacturer's instructions and densitometrically evaluated. Results are expressed as percentages of maximal TGFβ stimulation and are means ± SD of two independent experiments.

SPC suppresses IL-1β-induced inflammatory mediator production

In a next step, we investigated whether SPC was able to mimic TGFβ-mediated cell responses. TGFβ is well known to exert various biological effects, including an immunosuppressive effect, an anti-inflammatory effect, and a profibrotic effect, depending on the cell system. Previously, it was shown that in mesangial cells, TGFβ suppresses important proinflammatory enzyme systems such as the $sPLA_2$ or the inducible nitric oxide synthase. Stimulation of mesangial cells for 24 h with IL-1β led to a large increase of PGE_2 formation (**Fig. 5A**). In the presence of SPC, IL-1β-induced PGE_2 generation was partially suppressed. A significant effect (50% inhibition) was already seen at very low concentrations of 1 nM SPC, which was not further reduced by increasing the concentration to 10 μM. IL-1β-stimulated nitric oxide release was also reduced by SPC, but with a completely different potency compared with PGE_2 (Fig. 5B), which may suggest a different mode of action of SPC in the regulation of nitric oxide release.

To further elucidate the effect of SPC on PGE_2 formation, the expression of the IIA $sPLA_2$ was studied. Protein expression of IIA $sPLA_2$ in the supernatant, which was highly induced by IL-1β, was dose-dependently blocked by SPC (**Fig. 6A**). A similar reducing effect was also seen for the mRNA expression of IIA $sPLA_2$ (Fig. 6B), whereas the housekeeping enzyme GAPDH was not affected by either IL-1β or SPC (Fig. 6B).

Subsequently, the mechanism by which SPC can block $sPLA_2$ mRNA expression was investigated. mRNA steady-state levels may be reduced by two possible mechanisms: either by a reduced promoter activity or by increased mRNA degradation. To this end, we performed promoter studies. A 2.67 kb fragment of the rat $sPLA_2$ promoter was cloned and fused to a luciferase-containing vector (24). Transfection of mesangial cells with this $sPLA_2$ promoter fragment followed by stimulation for 24 h with IL-1β led to increased promoter activity (**Fig. 7**), confirming previous reports (24, 28). The cytokine-induced promoter activation was significantly, although only partially, reduced in the presence of SPC, resembling the partial effect of SPC on $sPLA_2$ mRNA expression seen in Fig. 6B. TGFβ was a more potent suppressor and completely abolished $sPLA_2$ promoter activity (Fig. 7). Moreover, the effect of SPC on $sPLA_2$ mRNA degradation was measured and was not affected by SPC (data not shown).

Furthermore, we determined whether this reducing effect of SPC on IIA $sPLA_2$ expression also involved the TGFβ/Smad cascade. To this end, cells were pretreated with the specific kinase inhibitor of the transforming growth factor-β receptor type I kinase (TKI). As seen in **Fig. 8A**, the suppressing effect of SPC on IL-1β-stimulated $sPLA_2$ protein expression was reversed in the presence of TKI. Similarly, SPC-suppressed IIA $sPLA_2$ promoter activity was also reversed by TKI (Fig. 8B).

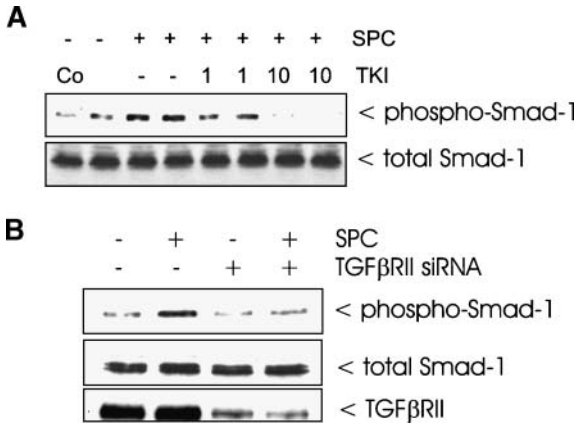

Fig. 4. Effects of TGFβ receptor types I and II (RI and RII) blockade on SPC-induced Smad phosphorylation. Quiescent mesangial cells were pretreated with vehicle [−; control (Co)] or the TGFβ RI inhibitor (10 μM; A) or TGFβ RII small interfering RNA (siRNA) (B) before SPC stimulation (3 μM) as described in Materials and Methods. Thereafter, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to Western blot analysis using specific antibodies against phospho-Smad-1 (1:1,000) and total Smad-1 (1:1,000). Bands were visualized by the ECL method according to the manufacturer's instructions. Data are representative of three independent experiments giving similar results. TKI, transforming growth factor-β receptor type I kinase inhibitor.

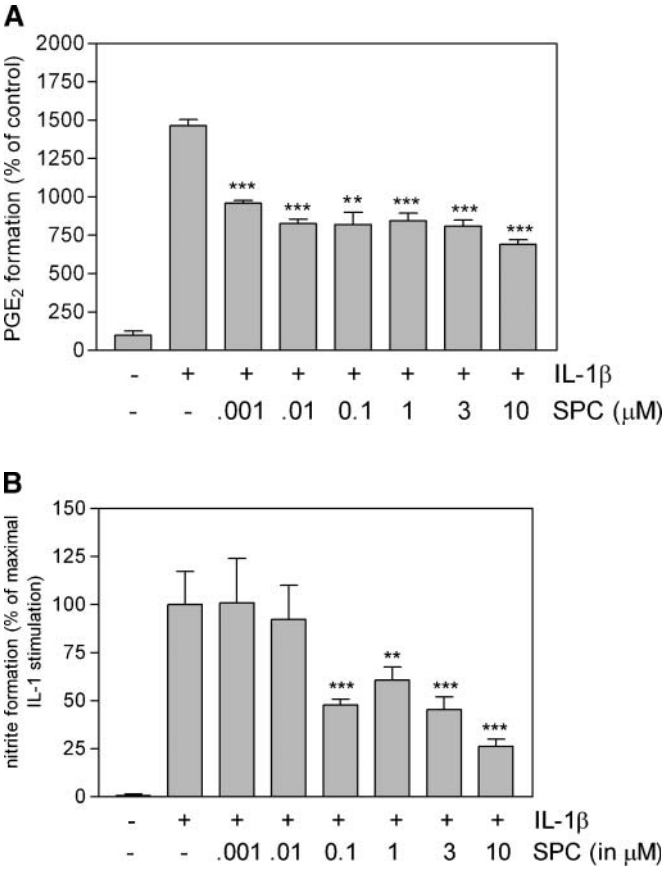

Fig. 5. Effects of SPC on interleukin-1β (IL-1β)-induced prostaglandin E_2 (PGE_2) and nitric oxide formation in mesangial cells. Quiescent mesangial cells were stimulated for 24 h with either vehicle (−) or IL-1β (1 nM; +) in the absence or presence of the indicated concentrations of SPC. Thereafter, supernatants were taken for the determination of PGE_2 formation (A) or nitrite formation (B) as described in Materials and Methods. Data are expressed as percentages of control and are means ± SD (n = 4). ** $P < 0.01$, *** $P < 0.001$, considered statistically significant compared with IL-1β-stimulated values.

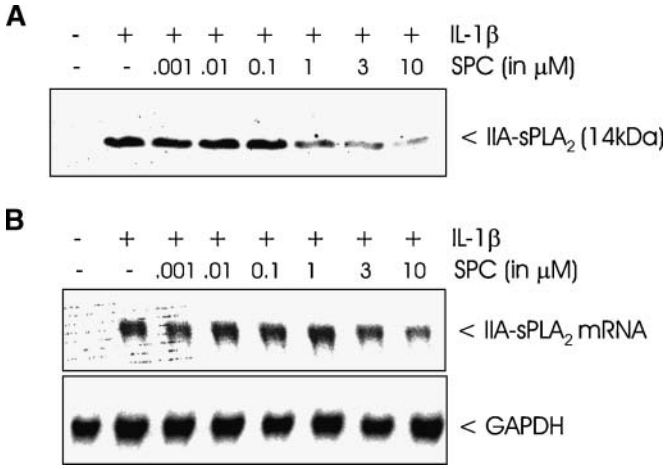

Fig. 6. Effects of SPC on IL-1β-induced secretory phospholipase A_2 ($sPLA_2$) mRNA and protein expression in mesangial cells. Quiescent mesangial cells were stimulated for 24 h with either vehicle (−) or IL-1β (1 nM; +) in the absence or presence of the indicated concentrations of SPC. Thereafter, supernatants were taken for protein precipitation followed by protein separation by SDS-PAGE, transfer to nitrocellulose, and Western blot analysis using a specific monoclonal antibody against rat IIA $sPLA_2$ at a dilution of 1:60 (A), or mRNA was extracted from the cell monolayer and subjected to Northern blot analysis using a specific probe for rat IIA $sPLA_2$ (B, upper panel) or GAPDH (B, lower panel). Bands in A were visualized by the ECL method according to the manufacturer's instructions. Bands in B were analyzed by an imaging system (Fuji). Data are representative of three independent experiments giving similar results.

Because TGFβ not only activates the regulatory Smad proteins but also induces the antagonistic Smad-7 (29), we investigated whether Smad-7 is upregulated by SPC and could account for the suppressive effect on PGE_2 formation. However, at up to 24 h of SPC stimulation, there was no expression of Smad-7 (data not shown).

To determine whether the reducing effect of SPC on cytokine-triggered PGE_2 synthesis also involves changes in COX-2 expression, we investigated the protein expression of COX-2. However, SPC did not significantly affect COX-2 protein expression (data not shown) suggesting that downregulation of the rate-limiting enzyme $sPLA_2$ is sufficient to reduce PGE_2 synthesis.

SPC-activated transcription of the profibrotic factor CTGF

In a final step, we investigated whether the profibrotic response classically triggered by TGFβ is also mimicked by SPC. One well-described profibrotic factor that is highly upregulated by TGFβ is the CTGF (30–32). Stimulation of mesangial cells with SPC caused a time-dependent increase of CTGF protein expression (**Fig. 9**). Already after 1 h, a clear upregulation was seen compared with vehicle-treated cells (Fig. 9). This, however, was no longer seen

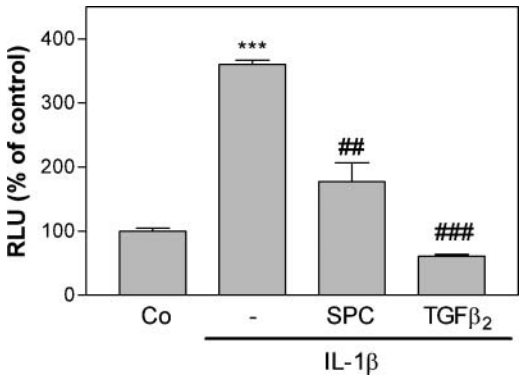

Fig. 7. Effects of SPC and $TGFβ_2$ on IL-1β-stimulated rat IIA $sPLA_2$ promoter activation. Mesangial cells were transfected with empty vector or a 2.67 kb fragment of the rat IIA $sPLA_2$ promoter plus the plasmid coding for Renilla luciferase. Twenty-four hours after transfection, cells were rendered serum-free and stimulated for 16 h with either vehicle [control (Co)] or IL-1β (1 nM) in the absence or presence of SPC (3 μM) or $TGFβ_2$ (10 ng/ml). The ratio between beetle and Renilla luciferase activities was calculated and is depicted as % of control relative luciferase activity [relative luciferase units (RLU)]. Data are means ± SD (n = 3). *** $P < 0.001$, considered statistically significant compared with vehicle-stimulated control values; ## $P < 0.01$, ### $P < 0.001$, considered statistically significant compared with IL-1β-stimulated control values.

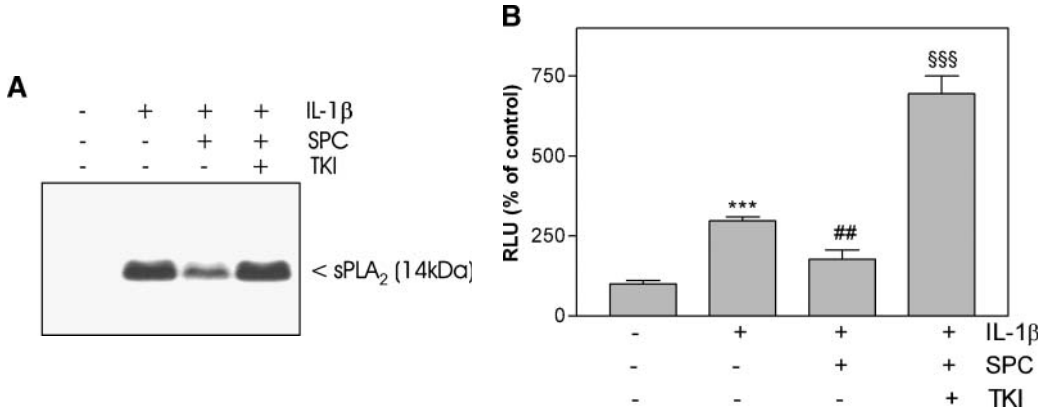

Fig. 8. Effects of TGFβ receptor inhibition on SPC-mediated inhibition of $sPLA_2$ protein expression and promoter activation in renal mesangial cells. A: Quiescent mesangial cells were stimulated with vehicle (−), IL-1β (1 nM), IL-1β + SPC (3 μM), or IL-1β + SPC + TKI (10 μM, pretreated for 20 min). Thereafter, supernatants were taken for protein precipitation followed by protein separation by SDS-PAGE, transfer to nitrocellulose, and Western blot analysis using a specific monoclonal antibody against rat IIA $sPLA_2$ antibody at a dilution of 1:60. B: Cells were transfected with the rat IIA $sPLA_2$ promoter plus the plasmid coding for Renilla luciferase. Twenty-four hours after transfection, cells were rendered serum-free and stimulated for 16 h with vehicle (−), IL-1β (1 nM), IL-1β + SPC (3 μM), or IL-1β + SPC + TKI (10 μM, pretreated for 20 min). The ratio between beetle and Renilla luciferase activities was calculated and is depicted % of control as relative luciferase activity [relative luciferase units (RLU)]. Data are means ± SD (n = 3). *** $P < 0.001$, considered statistically significant compared with vehicle-stimulated control values; ## $P < 0.01$, considered statistically significant compared with IL-1β-stimulated values; §§§ $P < 0.001$, considered statistically significant compared with IL-1β + SPC-stimulated values.

after 24 h, as a result of the high basal levels of CTGF produced in vehicle-treated cells. The stimulating effect of SPC on CTGF at 4 h was significantly blocked in the presence of the TGFβ RI kinase inhibitor (**Fig. 10A**). Additionally, depletion of the TGFβ RII by siRNA (Fig. 10B), or depletion of the downstream Smad-4 by siRNA (Fig. 10C), caused a marked, although only partial, reduction of SPC-induced CTGF protein expression.

DISCUSSION

In this study, we show for the first time that SPC displays an anti-inflammatory capacity in renal mesangial cells.

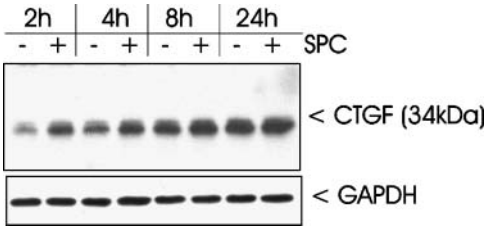

Fig. 9. Effects of SPC on connective tissue growth factor (CTGF) protein expression in renal mesangial cells. Cells were stimulated for the indicated time periods with either vehicle (−) or SPC (3 μM; +). Thereafter, protein lysates were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis using antibodies against CTGF (upper panel) or GAPDH (lower panel) at dilutions of 1:1,000 each. Bands were visualized by the ECL method according to the manufacturer's instructions. Data are representative of three independent experiments giving similar results.

Evidence is given by the fact that the expression of the IIA $sPLA_2$, which is considered a proinflammatory enzyme and affects the production of prostaglandins, is suppressed by SPC and consequently leads to a downregulation of PGE_2 synthesis. Mechanistically, this suppression of $sPLA_2$ mRNA expression is attributable to the activation of the TGFβ/Smad signaling cascade and critically involves the TGFβ receptor. This conclusion is based on the following findings: *i*) SPC stimulates the phosphorylation and activation of the Smad-1, -2, and -3 proteins (Fig. 2), which are blocked by a TGFβ receptor type I kinase inhibitor (Fig. 4A); *ii*) this TGFβ receptor type I kinase inhibitor is also able to reverse the reducing effect of SPC on $sPLA_2$ protein expression (Fig. 10A); and *iii*) depletion of the TGFβ receptor type II (Fig. 10B) or depletion of the downstream-located Smad-4 (Fig. 10C) by siRNA led to a reversal of the SPC effect. However, the activation of the TGFβ/Smad pathway by SPC occurs independent of TGFβ itself, as there is no increase of active TGFβ in the supernatant of SPC-treated cells when measured by ELISA (data not shown). Furthermore, neutralizing antibodies against TGFβ did not interfere with the SPC-triggered Smad phosphorylation (data not shown).

The finding that SPC stimulates Smad activation is in good agreement with our previous findings that S1P, and also the S1P mimetic FTY720 phosphate, are able to trigger the phosphorylation and activation of Smad proteins (33, 34). It is well established that S1P and FTY720 phosphate bind to the same receptors (35, 36) (i.e., the S1P receptors), and it has been proposed that there is a physical interaction between one of the S1P receptors, most likely $S1P_3$, and the TGFβ receptor (33). This was shown

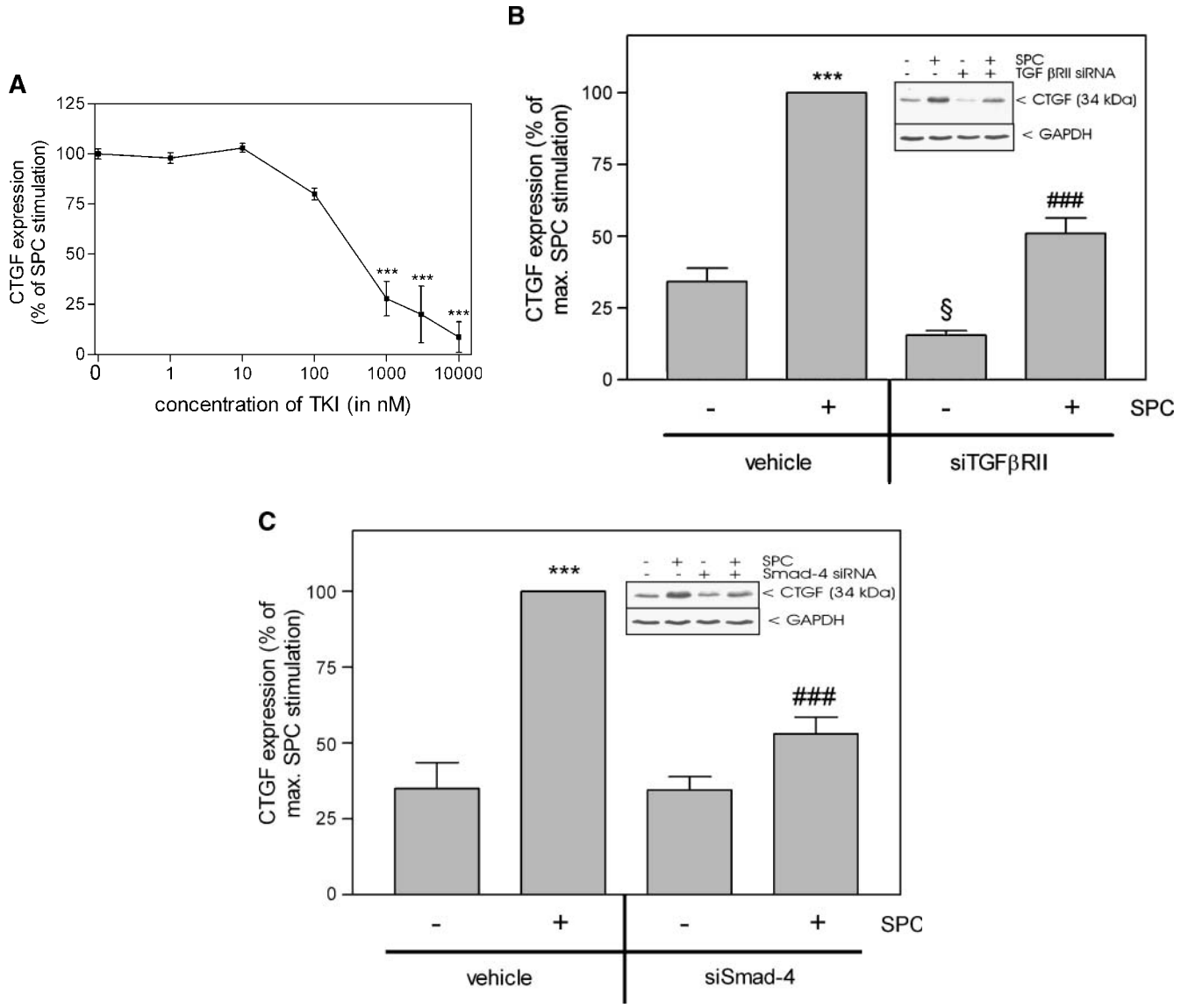

Fig. 10. Effects of TGFβ receptor inhibition and TGFβ RII and Smad-4 depletion by siRNA transfection on SPC-induced CTGF protein expression in mesangial cells. A: Cells were stimulated with vehicle or pretreated with the indicated concentrations of TKI before stimulation for 4 h with SPC (3 μM). B, C: Cells were transfected with vehicle, siRNA of TGFβ RII (B), or siRNA of Smad-4 (C) as indicated before stimulation for 4 h with either vehicle (−) or SPC (3 μM; +). Thereafter, protein lysates were prepared and separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis using antibodies against CTGF (insets, upper panels) or GAPDH (insets, lower panels) at dilutions of 1:1,000. Bands were visualized by the ECL method according to the manufacturer's instructions and were densitometrically evaluated. Results are expressed as percentages of maximal SPC stimulation and are means ± SD (n = 4). *** $P <$ 0.001, considered statistically significant compared with vehicle-stimulated control values; ### $P < 0.001$, considered statistically significant compared with vehicle-transfected and SPC-stimulated values; § $P < 0.001$, considered statistically significant compared with vehicle transfected unstimulated values.

using chemical cross-linkers, which resulted in an association of several factors with the TGFβ receptor type II upon S1P stimulation of mesangial cells (33). Whether SPC binds to the same receptors as S1P in mesangial cells remains unclear. Several in vitro studies showed that SPC can bind with low affinity to the S1P receptors. However, recently, high-affinity SPC receptors, such as ovarian cancer OGR1 (or GPR68), G2A (derived from G2 accumulation), and GRP4, were identified. Interestingly, these putative high-affinity SPC receptors are expressed, at least on the mRNA level, in mesangial cells (data not shown) and may mediate some of the SPC-triggered effects. In view of the concentration dependence of the activation of the MAPKs and Smad proteins, which is already seen at low nanomolar concentrations of SPC (Figs. 1B, 2B), one may speculate that one of the high-affinity SPC receptors is involved. However, it must be noted that not only S1P- but also SPC-induced vasodilation in preconstricted aortae is completely abolished in $S1P_3$-deficient mice, suggesting the involvement of the $S1P_3$ receptor in the SPC action on the endothelium (37).

There is now more evidence accumulating that G protein-coupled receptors undergo not only homodimerization

but also heterodimerization and oligomerization with other receptor classes, thereby triggering cross-talk between different signaling cascades (38). Also, this consideration makes it tempting to speculate that there is a direct interaction of the putative SPC receptor and the TGFβ receptor complex. In this regard, it has been shown that the S1P receptors can cross-activate other growth factor signaling cascades, such as vascular endothelial growth factor, epidermal growth factor, and platelet-derived growth factor receptor signaling (39–42).

The question of how Smad proteins negatively interfere with gene transcription is still largely unanswered. Although positive Smad binding elements (SBEs) in promoter regions of certain genes, mainly fibrosis-related genes such as CTGF (30–32), tissue inhibitor of metalloproteinases-1 (43), and plasminogen activator inhibitors (44, 45), are well reported, negative SBEs are seldom described. Ogawa et al. (46) first reported the existence of such a negative SBE-like sequence, denoted as transforming growth factor-β inhibitory element (TIE), in the human matrix metalloproteinase-9 promoter, although depletion and mutation studies revealed that this TIE is not involved in the TGFβ-mediated suppressive effect on matrix metalloproteinase-9 promoter activity (46). Recently, a novel suppressive SBE was identified within the TIE of the c-*myc* promoter, termed repressive SBE (47), which is different from the previously defined SBE and dictates the TGFβ-mediated repression of *c-myc* and subsequent growth arrest in keratinocytes (47, 48).

Alternatively, it has been suggested that Smad proteins exert a negative effect on gene transcription by indirect mechanisms such as interference with NFκB (46), AP-1 (49), c-Myc (50), NKX2.1 (51), and hepatocyte nuclear factor-3 (51), which prevents the binding of these transcription factors to their respective DNA binding sites.

The contribution of SPC to physiology and pathophysiology is poorly understood. Most consistently, a role of SPC in cardiovascular physiology is proposed based on the Ca^{2+}-mobilizing activity of SPC. Thus, in endothelial cells in situ on bovine aortic valves, SPC increases intracellular Ca^{2+} concentration and nitric oxide production and induces endothelium-dependent relaxation (52). In the smooth muscle of pig coronary arteries, SPC induces vessel contraction via Ca^{2+} sensitization (53). The vascular effects of SPC, and similarly of S1P, have gained further interest as a result of their association with HDL, which is a well accepted atheroprotective factor acting through various parallel protective mechanisms (37, 54). In the context of atherogenesis, SPC was also appointed an anti-inflammatory role as a result of its association with HDL, causing a reduction of the inhibitory effect on tumor necrosis factor-α-triggered NFκB activation, leading to reduced E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 expression (55).

In addition, SPC may have a relevant role in the regulation of the heart. Thus, SPC and S1P were identified as the plasma and serum factors responsible for activating the inwardly rectifying K^+ channel [I(K)], leading to a negative chronotropic effect (56).

SPC was also shown to be a potent mitogen for various cell types (27), and it was tempting to speculate that SPC could be involved in tumor development. Indeed, it was shown that SPC levels were significantly higher in ascites caused by ovarian cancer than in nonmalignant ascites (57). It was also proposed that SPC facilitates the metastasis of tumor cells by stimulation of a reorganization of intact keratin filaments, resulting in enhanced tumor cell elasticity (58). In contrast, another study proposed a role for SPC in tumor cell differentiation, as shown for human NB4 promyelocytic leukemia cells (59).

Furthermore, patients with atopic dermatitis have increased SPC and reduced ceramide levels in the skin, which correlate with an enhanced activity of a sphingomyelin (SM) deacylase in the skin of these patients (60–63). This suggests that SPC is formed by a phospholipase A_2 (PLA_2)-like enzymatic activity.

In general, little is known about the pathways of SPC generation and degradation. The SPC concentration in human serum was recently determined by mass spectrometry to be 130 ± 25 nM (56). Whether this is changed under inflammatory or fibrotic disease conditions has not been addressed. SPC formation is theoretically possible: *i*) from SM by the action of a PLA_2-like enzyme such as the SM deacylase (60, 61); *ii*) from sphingosine by phosphocholine transfer; or *iii*) from S1P by choline transfer. The generation of SPC from SM by PLA_2 activity is an intriguing hypothesis and is further stressed by the finding that cells isolated from Niemann-Pick patients, which possess an acid sphingomyelinase deficiency and consequently accumulate SM in the lysosomes, also show an accumulation of SPC (64). However, the mechanism of this phenomenon was not addressed further. In this context, several forms of lysosomal PLA_2 have been purified and characterized (65). Moreover, a lysosomal PLA_2 was recently cloned that possesses 1-*O*-acylceramide synthase activity (66). Whether one of these PLA_2s also possesses selectivity for SM to generate SPC has not been addressed.

No evidence is available at present to show that choline or phosphocholine transfer may play a role in SPC generation. On the other hand, the conversion of SPC to S1P may be catalyzed by a phospholipase D-like activity. Interestingly, autotaxin, a member of the nucleotide pyrophosphatase/phosphodiesterase family possessing lysophospholipase D activity, is able to hydrolyze LPC to form lysophosphatidic acid (67) as well as SPC to form S1P (68). Whether the conversion of SPC to S1P by autotaxin is of physiological or pathophysiological relevance remains unknown. At least autotaxin was shown to be an absolutely critical enzyme for life, as autotaxin-deficient mice are not viable and die at embryonic day E9.5 from profound vascular defects (69).

In summary, our study clearly shows that TGFβ and SPC share the same signaling device (i.e., the activation of the Smad signaling cascade), which strongly resembles the overlapping signaling between TGFβ and S1P. Furthermore, SPC not only mimics the anti-inflammatory effect of TGFβ on mesangial cells to suppress cytokine-induced PGE_2 formation and nitric oxide release but also exerts a

profibrotic effect, as shown by the upregulation of CTGF. In this way, SPC may represent a novel target for therapeutic strategies to cope with chronic inflammatory and fibrotic diseases.

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