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Substance P Stimulates Cyclooxygenase-2 and Prostaglandin E_2 Expression through JAK-STAT Activation in Human Colonic Epithelial Cells[1]

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Substance P (SP) via its neurokinin-1 receptor (NK-1R) regulates several gastrointestinal functions. We previously reported that NK-1R-mediated chloride secretion in the colon involves formation of PG. PGE_2 biosynthesis is controlled by cyclooxygenase-1 (COX-1) and COX-2, whose induction involves the STATs. In this study, we examined whether SP stimulates PGE_2 production and COX-2 expression in human nontransformed NCM460 colonocytes stably transfected with the human NK-1R (NCM460-NK-1R cells) and identified the pathways involved in this response. SP exposure time and dose dependently induced an early (1-min) phosphorylation of JAK2, STAT3, and STAT5, followed by COX-2 expression and PGE_2 production by 2 h. Pharmacologic experiments showed that PGE_2 production is dependent on newly synthesized COX-2, but COX-1 protein. Inhibition of protein kinase Cθ (PKCθ), but not PKCϵ and PKCδ, significantly reduced SP-induced COX-2 up-regulation, and JAK2, STAT3, and STAT5 phosphorylation. Pharmacological blockade of JAK inhibited SP-induced JAK2, STAT3, and STAT5 phosphorylation; COX-2 expression; and PGE_2 production. Transient transfection with JAK2 short-interferring RNA reduced COX-2 promoter activity and JAK2 phosphorylation, while RNA interference of STAT isoforms showed that STAT5 predominantly mediates SP-induced COX-2 promoter activity. Site-directed mutation of STAT binding sites on the COX-2 promoter completely abolished COX-2 promoter activity. Lastly, COX-2 expression was elevated in colon of mice during experimental colitis, and this effect was normalized by administration of the NK-1R antagonist CJ-12,255. Our results demonstrate that SP stimulates COX-2 expression and PGE_2 production in human colonocytes via activation of the JAK2-STAT3/5 pathway. ***The Journal of Immunology,*** **2006, 176: 5050–5059.**

Substance P (SP),[3] an 11-aa peptide member of the tachykinin family isolated by Leeman and Chang (1), is localized in the CNS as well as in peripheral tissues, including the intestine (2). SP is expressed in enteric nerves (3), sensory neurons (4), and macrophages of the intestinal lamina propria (5). Binding of SP to its high-affinity neurokinin-1 receptor (NK-1R) modulates important intestinal responses, such as mucosal permeability (6), colonic motility (7), chloride secretion (8), and acute intestinal inflammation (9, 10), via activation of the NF-κB (11, 12) and NF-κB-regulated cytokines (13, 14). SP-NK-1R interactions also induce proliferation of colonic epithelial as well as nonepithelial cells that involves activities of metalloproteinases and *trans* activation of the epidermal growth factor receptor (EGFR) (15, 16). This NK-1R-EGFR pathway appears to be also involved in the protective effects of NK-1R in regeneration and mucosal healing during chronic experimental colitis (17).

PGs represent a family of lipid mediators localized in the small intestine and colon and involved in various intestinal functions, including inflammation (18), cancer (19), and mucosal repair (20). Biosynthesis of PGs is mediated primarily by the rate-limiting enzymatic activities of cyclooxygenase (COX). COX comprise three categories, including COX-1, COX-2, and COX-3 (21). COX-1 is constitutively expressed in many cell types (22), while COX-2 is not avidly expressed under normal conditions, but it is induced in response to several stimuli (23). COX-3 is a newly discovered, paracetamol-inhibited, COX isoform that appears to be a splicing variant of COX-1 (24). Several pieces of evidence indicate that PGs might be associated with several SP-related responses. For example, in murine microglia, SP augments *Borrelia burgdorferi*-induced PGE_2 production (25), while a specific COX-2 inhibitor reduces spinal SP-evoked PGE_2 release (26). SP, at supraphysiologic concentrations (μM), stimulates PGE_2 release from rat intrapulmonary bronchi and trachea preparations via NK-1R activation (27, 28). Moreover, release of PGE_2 is involved in SP-induced relaxation of rat trachea (28), and mouse tracheal preparations via activation of PGE_2 receptor subtype 2 receptors (29).

The interaction(s) between SP and PGs in the colon or small intestine has not been extensively studied. Using Ussing chambers, we have reported previously that SP-NK-1R-mediated chloride secretion in human or animal colon involves formation of PGs (8, 30). PGE_2 exposure to guinea pig colonic myenteric ganglia alters electrical properties of myenteric neurons, and these effects are suppressed by a NK-1R antagonist (31). Moreover, Hosoda et al.

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[3] Abbreviations used in this paper: SP, substance P; CAPE, caffeic acid phenethyl ester; COX, cyclooxygenase; DSS, dextran sodium sulfate; EGFR, epidermal growth factor receptor; GAS, IFN-γ-activated sequence; NK-1R, neurokinin-1 receptor; PKC, protein kinase C; siRNA, short-interferring RNA; TFA, trifluoroacetic acid.

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(32) reported that PGE_2 acts synergistically with SP to enhance SP-evoked chloride secretion in guinea pig distal colonic epithelial cells. However, whether SP-NK-1R interactions can activate COX gene expression and stimulate PG production in colonocytes has not been examined, and the COX isoform(s) activated in response to SP is not clearly understood. Because NK-1R expression is upregulated in several forms of intestinal inflammation (33–36), including on colonic epithelial cells (37, 38), we used nontransformed human colonic epithelial NCM-460 cells overexpressing NK-1R to investigate whether SP activates COX-2 and PGE_2 secretion in colonocytes and examined the signal transduction pathways involved in this response. Our results demonstrate that SP can directly activate COX-2 expression and PGE_2 secretion in colonocytes. We also present novel evidence that SP activates the JAK-STAT pathway that is critical for SP-induced COX-2 gene expression and PGE_2 production.

Materials and Methods

Cell cultures

The nontransformed human colonic epithelial NCM460 cells overexpressing NK-1R (NCM460-NK-1R) have been previously described by us (13), and used in studies investigating both proinflammatory (13, 14) and cell proliferative responses (16) to SP. Cells were cultured in M3D medium (INCELL) containing 10% FCS (Invitrogen Life Technologies) and 1% penicillin/streptomycin (Invitrogen Life Technologies) solution.

Pharmacological experiments

NCM460-NK-1R cells were seeded in 12-well plates (2×10^6 cells/plate) overnight in M3D medium containing 10% FCS and 1% penicillin/streptomycin (Invitrogen Life Technologies) and then serum starved for 24 h. Cells were pretreated with NS-398 (60 μM), indomethacin (20 μM), FR122047 (20 μM), cycloheximide (50 μM), JAK inhibitor I (10–40 μM), SB203580 (20 μM), AG1478 (2 μM), rottlerin (mallotoxin 0.1–1 μM), caffeic acid phenethyl ester (CAPE, 20 μM), specific protein kinase Cθ (PKCθ) pseudosubstrate peptide inhibitor (Myr-LHQRRGAIKQA KVHHVKC-NH_2 10 μM), PKCϵ pseudosubstrate peptide inhibitors (EAVSLKPT 10 μM) (Calbiochem), and CJ-12,255 (0.1–1 μM) (generously provided by Pfizer), 30 min before SP or 0.1% trifluoroacetic acid (TFA) (vehicle control) for various time points.

Mice colon tissue preparation

Male 8- to 10-wk-old C57BL/6 mice (n = 6/group) were purchased from Charles River Laboratories and were maintained at the animal research facility of Beth Israel Deaconess Medical Center under standard environmental conditions. Mice received standard pelleted chow and tap water ad libitum, except the colitis group, which received water containing dextran sodium sulfate (DSS) 5% (w/v), as previously described (17). To test the participation of NK-1R, mice were injected i.p. with 200 μl of PBS containing the specific NK-1R antagonist CJ-12,255 (2.5 mg/kg/twice per day), or PBS alone. After 5 days, mice were sacrificed by carbon dioxide euthanasia. Colon tissues were dissected and homogenized in radioimmunoprecipitation buffer (Santa Cruz Biotechnology), and equal amounts of protein (40 μg/lane) were loaded for Western blot experiments.

PGE_2 ELISA

PGE_2 levels in conditioned medium were determined by ELISA kits (R&D Systems), according to the manufacturer's instructions. Results were expressed as mean ± SEM (pg/ml).

Western blot analyses

SP-treated cells were lysed in 1× lysis buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.01% bromphenol blue, and 1% 2-ME). Equal amounts of cell extracts were fractionated by 10% SDS-PAGE, and proteins were transferred onto nitrocellulose membranes (Bio-Rad) at 400 milliamps for 2 h at 4°C. Membranes were blocked in 5% nonfat milk in TBST (50 mM Tris (pH 7.5), 0.15 M NaCl, 0.05% Tween 20), and then incubated with Abs against phospho-JAK1 ($Tyr^{1022/1023}$), phospho-JAK2 ($Tyr^{1007/1008}$), phospho-STAT1 (Tyr^{701}), phospho-STAT3 (Tyr^{705}), phospho-STAT5 (Tyr^{694}), phospho-STAT6 (Tyr^{641}) (Cell Signaling Technology), phospho-JAK3 (Tyr^{980}), COX-1, COX-2 (Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich). HRP-labeled Abs were detected by ECL (Pierce). The image of the signal was exposed to x-ray film (Fujifilm). In some experiments, Western blot bands were quantified by densitometry and Scion image analysis software with normalization of the phosphorylated protein or nonphosphorylated protein bands to the corresponding band of control (β-actin) signal from the same samples.

COX-2 promoter luciferase assays

A 2004-bp-long COX-2 promoter region spanning −2069 to −66 bp upstream of the translational start site was cloned by PCR and subcloned into pGL3 vector (pGL3-Cox-2). The sequence was confirmed by DNA sequencing analysis. NCM460-NK-1R cells were seeded in 12-well plates (2×10^6 cells/plate) overnight and transiently cotransfected with pGL3-Cox-2 along with an internal control pRL-TK (Promega) and/or siRNA for JAK1, JAK2 (Upstate Biotechnology), STAT3, STAT5, STAT6, or control short-interferring RNA (siRNA) (Santa Cruz Biotechnology) using LipofectAMINE 2000 transfection reagent (Invitrogen Life Technologies), following the manufacturers' instructions. Transfected cells were serum starved for 24 h, followed by SP exposure for 4 h. Firefly and *Renilla* luciferase activities in cell extracts were measured using a dual-luciferase reporter assay system (Promega). The relative luciferase activity was then calculated by normalizing COX-2 promoter luciferase activity to control *Renilla* luciferase activity. Results are expressed as percentage of relative luciferase activity of the control group without SP stimulation, which was set as 100%.

Site-directed mutagenesis of the STAT binding sites of the COX-2 promoter

The wild-type COX-2 promoter used in the above luciferase assays was modified by Promega's GeneEditor in vitro site-directed mutagenesis kit

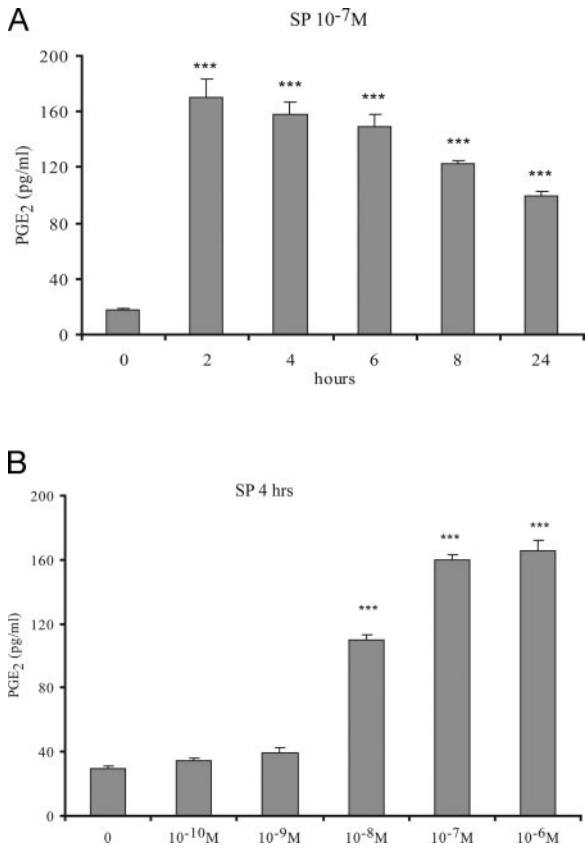

FIGURE 1. SP stimulates PGE_2 secretion in NCM460-NK-1R colonocytes expressing NK-1R. Serum-starved NCM460-NK-1R cells were treated with SP (10^{-7} M) for the indicated time points (*A*) or with different doses (0–10^{-6} M) of SP for 4 h (*B*). Conditioned medium was collected, and PGE_2 levels were measured by ELISA. SP induces significant PGE_2 release in time-dependent and dose-dependent manner. Data are expressed as mean ± SEM and are representative of six independent samples. ***, Indicates statistical significant difference $p < 0.001$ vs control group of (*A*) time 0 min and (*B*) SP 0 M, respectively.

(Promega catalogue Q9280). Two STAT-binding elements, namely IFN-γ-activated sequence (GAS) motif sequences COX-2-sp3 (5′-TCTCTTTC CAAGAAACAAG-3′ from −895 to −877) and COX-2-sp4 (5′-ATT TCTTCTGTTGAAAGCAA-3′ from −850 to −831), were modified to 5′-TCTCTCCCCAAAAAACAAG-3′ (sp3) and 5′-ATTTCCCCTGTTA AAAGCAA-3′ (sp4), respectively. Underlined sequences denote the GAS motifs (sp3 and sp4). The mutated COX-2 promoter constructs (pGL3-Cox-2-sp3 mut and pGL3-Cox-2-sp4 mut) as well as the pGL3-Cox-2 were transfected into cells, as described above. Luciferase assays were performed to measure its influence on SP-induced COX-2 promoter activities.

Statistical analyses

ELISA and luciferase assay results were analyzed using Prism professional statistics software program (GraphPad). ANOVA were used for intergroup comparisons.

Results

SP stimulates PGE_2 production in colonocytes in a time- and dose-dependent manner

To start investigating whether SP causes PGE_2 production in colonocytes, NCM460-NK-1R cells were treated with SP (10^{-7} M) for the indicated time points, and conditioned medium was collected for PGE_2 ELISA. SP induced a rapid release of PGE_2 as early as 2 h after SP exposure, remained at high levels for 6 h (Fig. 1*A*), but slowly reduced at 8 and 24 h, albeit at higher levels than controls (Fig. 1*A*). The response of PGE_2 production within 4 h was dose dependent, with detectable SP induction at 10^{-8} M (Fig. 1*B*). In all subsequent experiments, we used a submaximal dose of SP (10^{-7} M).

SP-induced PGE_2 production depends on NK-1R, de novo synthesis of COX-2, and activation of the JAK pathway

To determine which COX isoform is involved in SP-induced PGE_2 production, NCM460-NK-1R cells were pretreated with the COX-2-specific inhibitor NS-398 (60 μM), or the COX-1/2 inhibitor indomethacin (20 μM), the COX-1-specific inhibitor FR122047 (20 μM), or the NK-1R antagonist CJ-12,255 (1 μM), 30 min before SP stimulation for 4 h. SP-induced PGE_2 was completely inhibited by NS-398, indomethacin, and CJ-12,255, but not FR122047 (Fig. 2*A*), indicating that PGE_2 production in response to SP is mediated by NK-1R and COX-2, but not COX-1. Pretreatment of colonocytes with the protein synthesis inhibitor cycloheximide (50 μM) led to diminished SP-induced PGE_2 levels (Fig. 2*B*), suggesting that de novo COX-2 protein synthesis is required for this response.

Prior studies demonstrated the importance of the JAK-STAT signal transduction pathway in PGE_2 production (39, 40). To investigate whether this pathway is involved in NK-1R-associated PGE_2 secretion, NCM460-NK-1R cells were pretreated with the JAK inhibitor, JAK inhibitor I (40 μM), for 30 min, followed by SP (10^{-7} M) stimulation for 4 h. Our results showed that JAK inhibitor I completely inhibited SP-induced PGE_2 production (Fig.

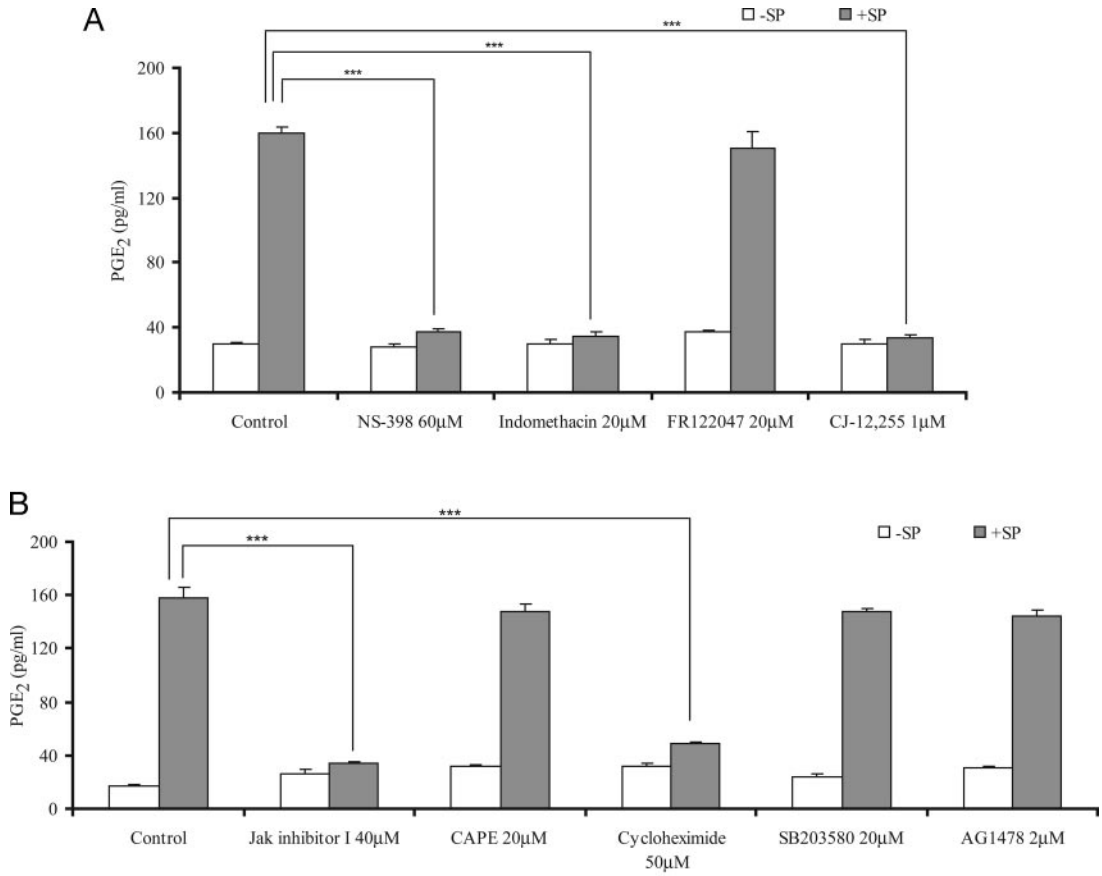

FIGURE 2. SP-induced PGE_2 secretion depends on JAK2 and COX-2, but not COX-1 expression. *A*, Serum-starved NCM460-NK-1R cells were pretreated with indomethacin (20 μM), NS-398 (60 μM), FR122047 (20 μM), or CJ-12,255 (1 μM) for 30 min, followed by TFA or SP (10^{-7} M) for 4 h. *B*, Serum-starved NCM460-NK-1R cells were pretreated with JAK inhibitor I (40 μM), CAPE (20 μM), cycloheximide (50 μM), SB203580 (20 μM), or AG1478 (2 μM) for 30 min, followed by TFA or SP (10^{-7} M) exposure for 4 h. Conditioned cell medium was collected, and PGE_2 levels were measured by ELISA. PGE_2 release is dependent on de novo COX-2 protein synthesis and JAK pathways. COX-1 does not appear to mediate SP-induced PGE_2 synthesis. Data are expressed as means ± SEM and are representative of six independent samples. ***, Indicates $p < 0.001$ vs control group.

2*B*), indicating the NK-1R-mediated JAK signaling is essential for SP-induced PGE_2 secretion.

Several reports demonstrated that SP-NK-1R binding stimulates p38 expression (41), activates NF-κB (11, 13, 14), and *trans* activates the EGFR (15, 16). However, pretreatment of NCM460-NK-1R cells with the NF-κB inhibitor CAPE (20 μM), the p38 inhibitor SB203580 (20 μM), or the EGFR inhibitor AG1478 (2 μM) did not affect SP-induced PGE_2 secretion (Fig. 2*B*), indicating lack of involvement of these NK-1R-related signaling pathways in SP-induced PGE_2 synthesis.

SP induces COX-2 expression in a time- and dose-dependent manner

PGs, including PGE_2, are the enzymatic products of COX. Because our results indicated that COX-2, but not COX-1, mediates SP-induced PGE_2 secretion, we examined the expression of COX-2 by Western blotting. We found that COX-2 protein expression was increased as early as 2 h after SP exposure (Fig. 3*A*), remained elevated after 4–6 h, and then started to decline at 8 and 24 h ($p < 0.001$) (Fig. 3*A*). This SP-induced COX-2 time-dependent expression was correlated with the PGE_2 secretion pattern shown in Fig. 1*A*. SP also substantially increased COX-2 expression ($p < 0.001$) in a dose-dependent manner with detectable induction at 10^{-8} M, and higher induction at 10^{-7} and 10^{-6} M (Fig. 3*B*). Under the same conditions, however, COX-1 protein was undetectable in all samples from SP-exposed colonocytes (data not shown).

SP-induced COX-2 expression is NK-1R dependent

As shown in Fig. 2*A*, SP-induced PGE_2 was dependent on NK-1R. In this study, we pretreated NCM460-NK-1R cells with the NK-1R antagonist CJ-12,255 (1 μM), before SP (10^{-7} M) stimulation for 4 h, and then performed Western blot analysis. We found that CJ-12,255 completely blocked SP-induced COX-2 expression (Fig. 3*C*). To examine the pathophysiologic significance of this response in vivo, we injected CJ-12,255 (5 mg/kg/day i.p.) into C57BL/6 mice during 5 days, DSS-induced colitis period. We found that, as expected (42), basal levels of COX-2 in colonic tissues of non-DSS-treated mice were detectable, while DSS treatment induced colonic inflammation, as we previously reported (17), which was associated with significant up-regulation of COX-2 (by ~2-fold; $p < 0.001$) (Fig. 3*D*). However, administration of CJ-12,255 significantly reduced COX-2 levels to levels seen in control, non-DSS-exposed mice ($p < 0.001$; Fig. 3*D*).

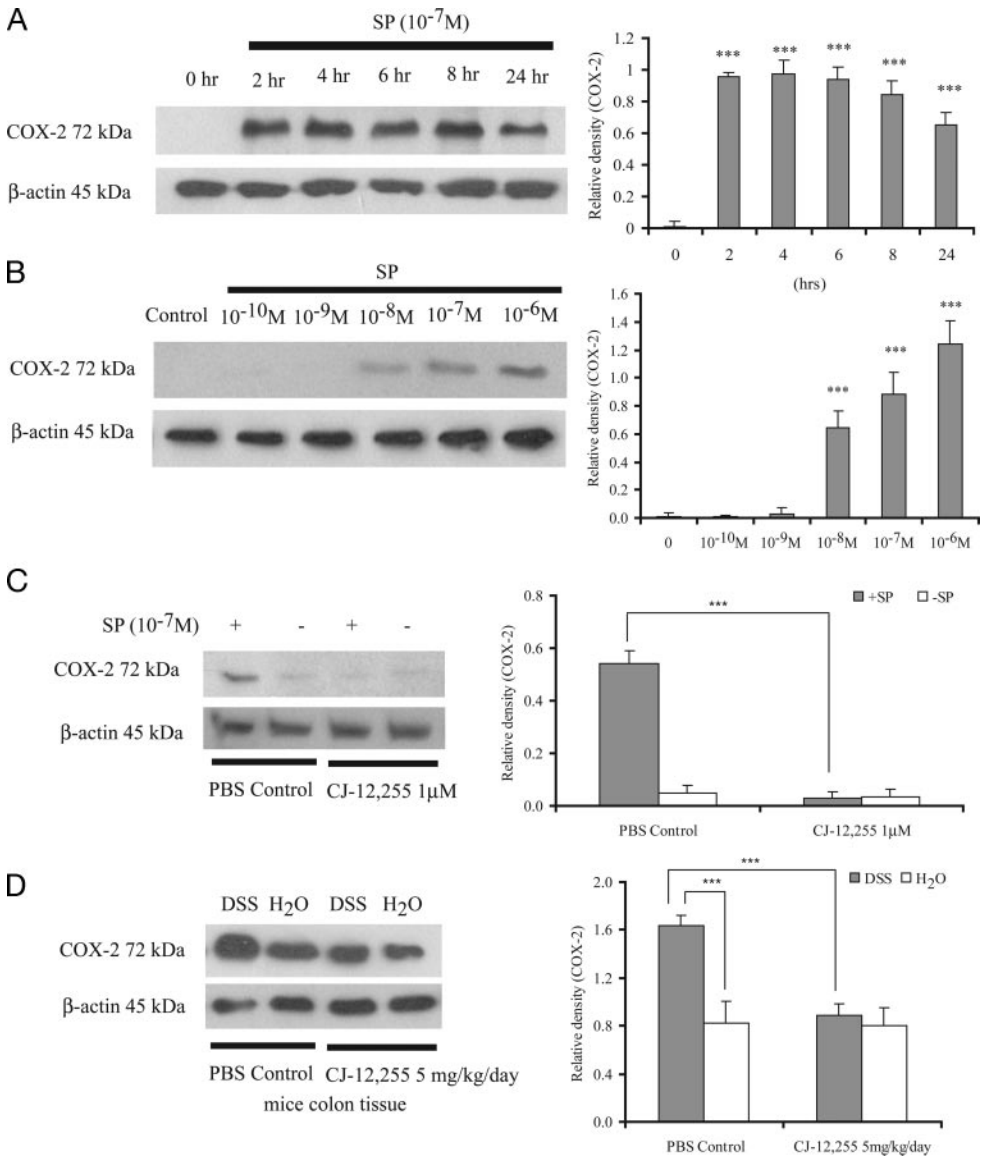

FIGURE 3. SP induces COX-2 expression in a time- and dose-dependent manner. Serum-starved NCM460-NK-1R cells were treated with SP (10^{-7} M) for the indicated time points (*A*) and with different doses (0–10^{-6} M) of SP for 4 h (*B*). Serum-starved NCM460-NK-1R cells were pretreated with NK-1R antagonist CJ-12,255 (1 μM) for 30 min, followed by TFA or SP (10^{-7} M) exposure for 4 h (*C*). Male C57BL/6 mice were provided with 5% DSS solution or H_2O to induce colitis, with/without CJ-12,255 (5 mg/kg/day) i.p. injections for 5 days; colon tissues were then dissected for protein extraction (*D*). Tissues were homogenized, and equal amounts of protein were fractioned on 10% SDS-polyacrylamide gels to determine the levels of COX-2 protein expression. Densitometric analyses of Western blot experiments were shown on the *right-hand side* of the images. Results were representative of three independent experiments (*A*–*C*) and six mice per group (*D*).

Together with the results shown in Fig. 3*C*, these findings indicate that SP-induced COX-2 expression requires NK-1R activation in vitro and in vivo.

SP-NK-1R induces phosphorylation of JAK-2, STAT3, and STAT5

As SP-induced PGE_2 production requires COX-2 expression that involves the JAK pathway, we examined whether SP activates signaling molecules upstream of COX-2 transcription, such as the members of the JAK and STAT family (40, 43). NCM460-NK-1R cells were stimulated with SP (10^{-7} M) and, at several time intervals, cells were lysed and equal amounts of protein were subjected to Western blot analysis using Abs directed against the phosphorylated forms of several COX-2-associated signal transduction molecules. Our data showed that SP, time dependently, induced phosphorylation of JAK2, STAT3, and STAT5, with activation evident as early as 1 min after exposure (Fig. 4*A*). SP-induced phosphorylation of JAK2, STAT3, and STAT5 was also dose dependent, with induction of JAK2 and STAT5 with SP concentrations as low as 10^{-10} M, while higher than 10^{-8} M SP concentrations were needed for STAT3 induction (Fig. 4*B*). In contrast, under the same conditions, SP failed to phosphorylate JAK1, JAK3, STAT1, or STAT6 (data not shown). Moreover, in agreement with data in Fig. 2*A* and Fig. 3, *C* and *D*, pretreatment with the NK-1R antagonist CJ-12,255 (1 μM) blocked SP-induced phosphorylation of JAK2, STAT3, and STAT5 in NCM460-NK-1R cells (Fig. 4*C*).

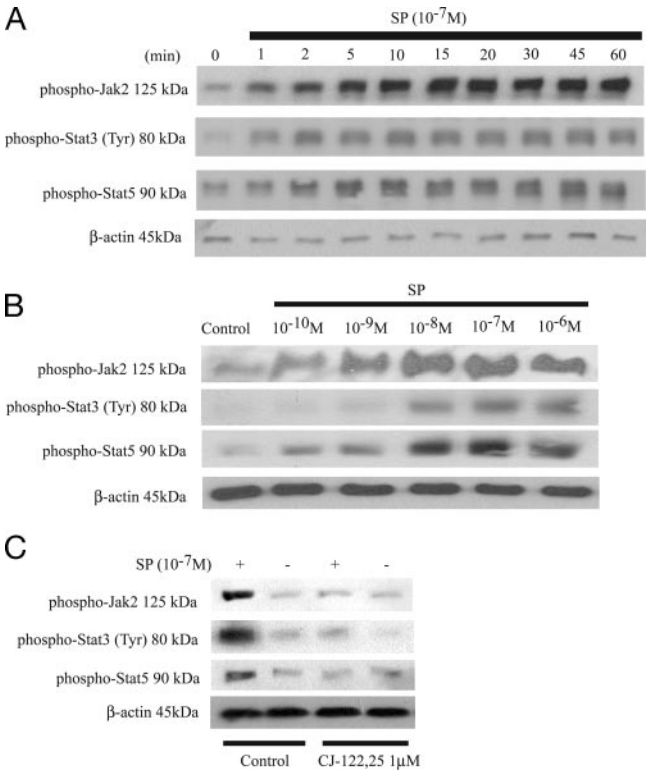

FIGURE 4. SP induces JAK2, STAT3, and STAT5 phosphorylation. Serum-starved NCM460-NK-1R cells were treated with SP (10^{-7} M) for the indicated time points (*A*) and with different doses (0–10^{-6} M) of SP for 20 min (*B*). Serum-starved NCM460-NK-1R cells were pretreated with NK-1R antagonist CJ-12,255 (1 μM) for 30 min, followed by TFA or SP (10^{-7} M) exposure for 20 min (*C*). Cells were lysed, and equal amounts of protein were fractioned on 10% SDS-polyacrylamide gels to determine the levels of phospho-JAK2, phospho-STAT3 (Tyr), phospho-STAT5, and β-actin. Results are representative of three independent experiments.

SP-induced COX-2 expression dependent on the PKCθ-JAK-STAT pathway

Next, we sought to identify the sequence of JAK2 and STAT activation following SP stimulation by both pharmacological and molecular approaches. NCM460-NK-1R colonocytes were pretreated with the JAK inhibitor I (0–40 μM) for 30 min and exposed to SP (10^{-7} M) for 20 min, and cell lysates were prepared and processed for Western blot analysis. Our results showed that JAK inhibitor I, dose dependently, inhibited SP-induced JAK2, STAT3, and STAT5 phosphorylation (Fig. 5*A*), indicating that SP-associated STAT3 and STAT5 phosphorylation requires JAK2 activity. Moreover, pretreatment with the JAK inhibitor I (0–40 μM) resulted in an inhibition of SP-induced COX-2 expression at 40 μM, but not at 10 μM (Fig. 5*B*), suggesting a link between the JAK2-STAT pathway and COX-2 expression in response to SP. Densitometric analyses demonstrated that only 40 μM JAK inhibitor I significantly blocked SP-induced COX-2 up-regulation ($p < 0.001$).

Evidence indicates that PKC activation may be linked to JAK-STAT phosphorylation (44, 45). We recently reported that SP induces PKCδ (δ), PKCθ (θ), and PKCϵ (ϵ) phosphorylation, and that only PKCδ mediates SP-induced IL-8 expression via NF-κB interaction (14). Therefore, we pretreated NCM460-NK-1R colonocytes with specific pseudosubstrate inhibitors directed against PKCθ and PKCϵ, as well as with rottlerin that inhibits PKCδ to detect their influence in COX-2 expression. The concentrations of these PKC inhibitors used in this study had also been used in our previous publication (14). We found that only the PKCθ pseudosubstrate inhibitor, but not inhibitors directed against PKCδ or PKCϵ, significantly reduced SP-induced JAK2, STAT3, and STAT5 phosphorylation (Fig. 5*C*) and COX-2 expression ($p < 0.001$) (Fig. 5*D*), suggesting that PKCθ is upstream of JAK2 signaling in response to SP.

JAK2 mediates SP-induced COX-2 promoter activity

To confirm the roles of JAK2 in COX-2 expression and PGE_2 production, we also examined the effect of JAK2 silencing by the siRNA approach in SP-induced COX-2 promoter activity. Transfection of siRNAs targeting JAK2 significantly inhibited SP-induced *COX-2* promoter activity (Fig. 6*A*). The siRNA inhibitory efficiencies were validated by Western blot analyses, demonstrating that JAK2 RNA silencing reduced JAK2 expression, and diminished SP-induced JAK2 phosphorylation, respectively (Fig. 6*B*). Because, as indicated above, SP did not induce JAK1 phosphorylation, we used siRNA targeting of JAK1 as a negative control. Our results show that JAK1 silencing did not influence SP-mediated COX-2 promoter activity (Fig. 6*A*). Densitometric analysis of the results from Fig. 6*B* showed that compared with control siRNA, JAK2 siRNA significantly inhibited SP-induced phosphorylated and nonphosphorylated JAK2 expression by 69 and 66%, respectively (Fig. 6, *C* and *D*).

STAT5 and STAT3 mediate SP-induced COX-2 activity

Because our results suggest that SP-induced STAT3 and STAT5 activation mediate COX-2 expression, we next sought to determine the relative contribution of these two isoforms in SP-induced COX-2 induction. We cotransfected NCM460-NK-1R colonocytes with siRNAs targeting either STAT3, STAT5, or a control siRNA together with a wild-type COX-2 promoter plasmid plus an internal control plasmid and measured their influence on COX-2 promoter activity. Silencing of STAT6, a STAT that as shown above is not phosphorylated by SP, served as another control. We found

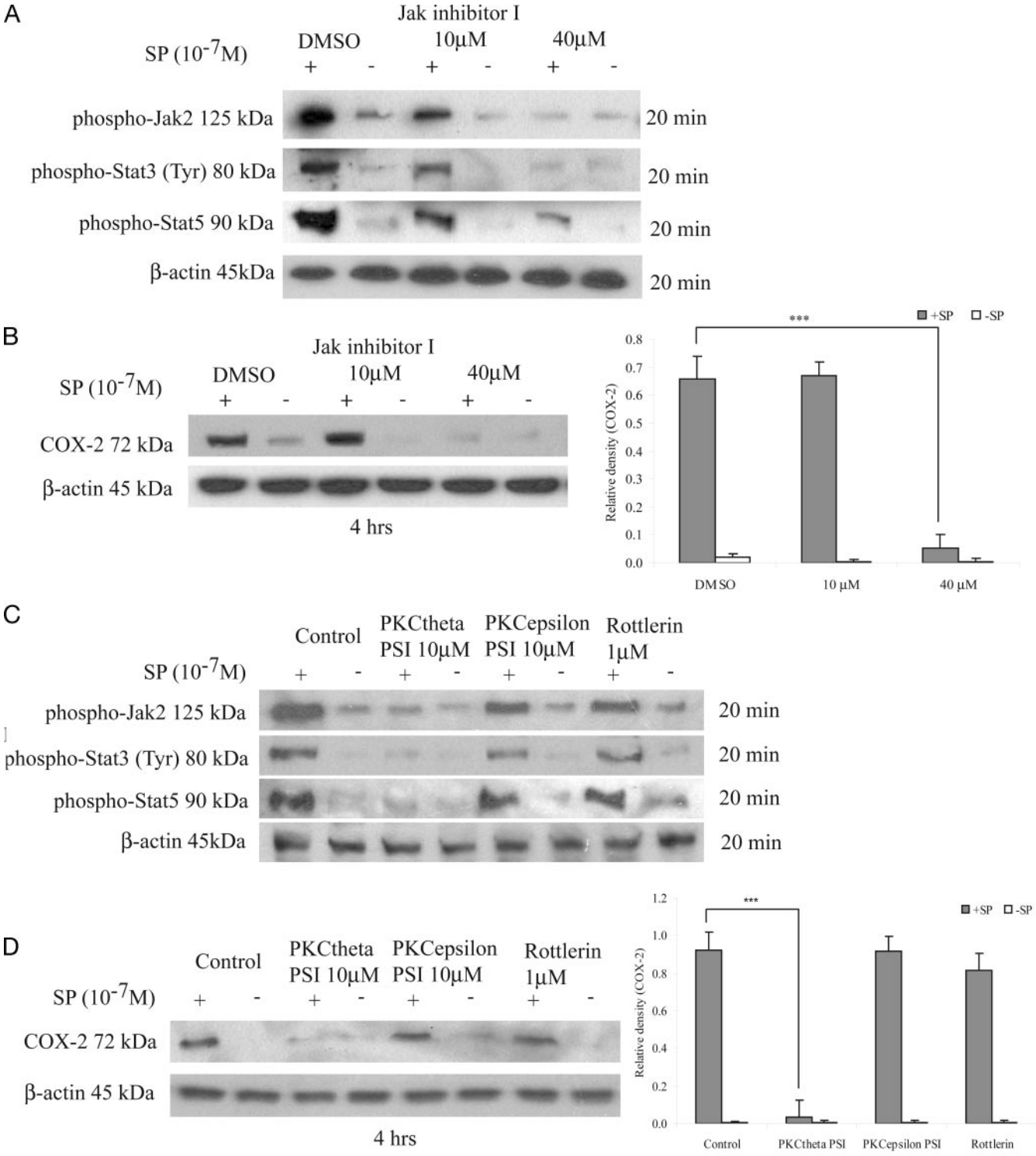

FIGURE 5. Pharmacological blockades of JAK and PKCθ inhibit SP-induced STAT3/5 phosphorylation and COX-2 expression. Serum-starved NCM460-NK-1R cells were pretreated with various doses of JAK inhibitor I or vehicle control DMSO for 30 min, followed by SP (10^{-7} M) exposure for 20 min (*A*) or 4 h (*B*). Serum-starved NCM460-NK-1R cells were pretreated with a PKCθ pseudosubstrate inhibitor (10 μM), a PKCϵ pseudosubstrate inhibitor (10 μM), rottlerin (1 μM), or vehicle control DMSO for 30 min, followed by SP (10^{-7} M) for 20 min (*C*) or 4 h (*D*). Cells were lysed, and equal amounts of protein were fractionated on 10% SDS-polyacrylamide gels to determine the levels of phospho-JAK2, phospho-STAT3 (Tyr), phospho-STAT5, COX-2, and β-actin. Results are representative of three independent experiments. Densitometric analyses of COX-2 Western blot experiments were shown on the *right-hand side* of the images.

that both STAT3 and STAT5 RNA interference significantly inhibited SP-induced COX-2 activity, while STAT6 RNA interference did not affect SP-induced *COX-2* activity (Fig. 7*A*). STAT5 siRNA exerted a more profound effect on SP-induced COX-2 activity than STAT3 siRNA on (Fig. 7*A*). To confirm that these effects were not related to a different level of inhibition of their

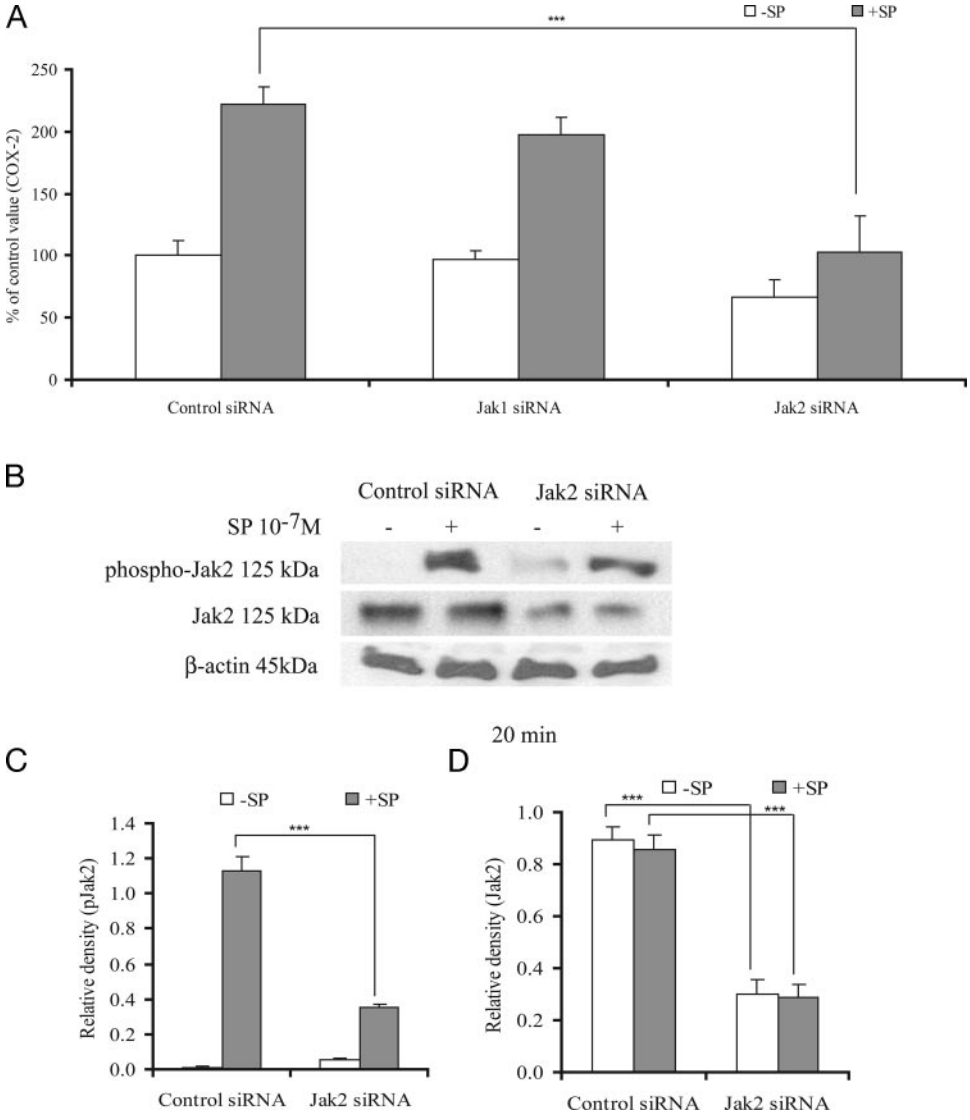

FIGURE 6. RNA interference of JAK2 inhibits COX-2 promoter transcriptional activity. *A*, NCM460-NK-1R cells were transiently transfected with siRNAs of JAK2 or negative control together with the wild-type COX-2 promoter luciferase reporter plasmid and an internal control pRL-TK plasmid. The transfected cells were serum starved overnight and treated with SP (10^{-7} M) for 4 h. Cell extracts were prepared to measure COX-2 promoter activity. Data are expressed as means ± SEM (percentage of relative firefly luciferase activity, normalized to control group without SP stimulation, which was normalized as 100%) and are representative of six independent samples. *B*, Control siRNA- or JAK2 siRNA-transfected cells were lysed, and equal amount of proteins was used to determine the levels of JAK-2, phospho-JAK2, and β-actin. Densitometric analyses of Western blot experiments with phospho-JAK2 (*C*) and JAK2 (*D*) indicated that JAK2 siRNA significantly inhibited JAK2 protein expression and SP-induced phospho-JAK2 signal. ***, Indicates $p < 0.001$ vs control group. Western blot results are representative of three independent experiments.

endogenous protein expression, we also validated the siRNA inhibitory efficiencies by Western blot analyses. Our results showed that both STAT3 and STAT5 siRNAs significantly inhibited SP-induced phospho-STAT3 (Fig. 7*B*) and phospho-STAT5 expression as well as their endogenous protein expression (Fig. 7*C*). Densitometric analyses of the results (Fig. 7*B*) showed that STAT3 siRNA significantly reduced the SP-induced phospho-STAT3 signal by 83% (Fig. 7*B*) and STAT3 signal by ~63% (Fig. 7*B*). Moreover, incubation with STAT5 siRNA significantly inhibited SP-induced phospho-STAT5 signal by 80% (Fig. 7*C*) and nonphosphorylated STAT5 signal by ~61% (Fig. 7*C*). Together, these results strongly suggest that STAT3 and STAT5 are important mediators of SP-mediated COX-2 activity.

SP-induced COX-2 promoter activity requires intact binding sequences of both GAS sites

Binding of STAT5 to the GAS motifs on the COX-2 promoter in human monocytes has been demonstrated previously (40). To confirm that the STAT pathway is essential for SP-induced COX-2 expression, we modified the two STAT binding sites (GAS sp3 and sp4 motifs) on the COX-2 promoter, as described in *Materials and Methods*, by site-directed mutagenesis. The wild-type and mutated COX-2 promoter constructs were then transiently transfected into NCM460-NK-1R cells before SP treatment and COX-2 promoter activity was determined. We found that mutation of either sp3 or sp4 site completely abolished SP-induced COX-2 promoter activities (Fig. 8). Basal *COX-2* promoter activity was also reduced in sp3- or sp4-mutated cells. These results point to an important role for both COX-2 promoter GAS sites in SP-induced COX-2 gene transcription in nontransformed human colonic epithelial cells.

Discussion

SP and its high-affinity NK-1R are major mediators of several intestinal responses, including chloride secretion and inflammation. Previous studies indicate that PGs might be involved in SP-mediated chloride secretion in the animal and human colon, by acting on cells of the intestinal mucosa (8, 30), as well as on guinea pig colonic epithelial cells (32). However, whether SP stimulates *COX* gene expression in human colonocytes is not known, and the COX isoform(s) activated in response to SP in these cells has not been identified. We report in this work for the first time that in nontransformed human colonocytes, SP, via NK-1R signaling, stimulates rapid COX-2, but not COX-1 expression and significant production of PGE_2. Moreover, significant COX-2 induction was evident in the colon of mice with DSS-induced colitis, and this effect was normalized in animals injected with a specific NK-1R antagonist CJ-12,255 (Fig. 3*D*), suggesting the pathophysiologic

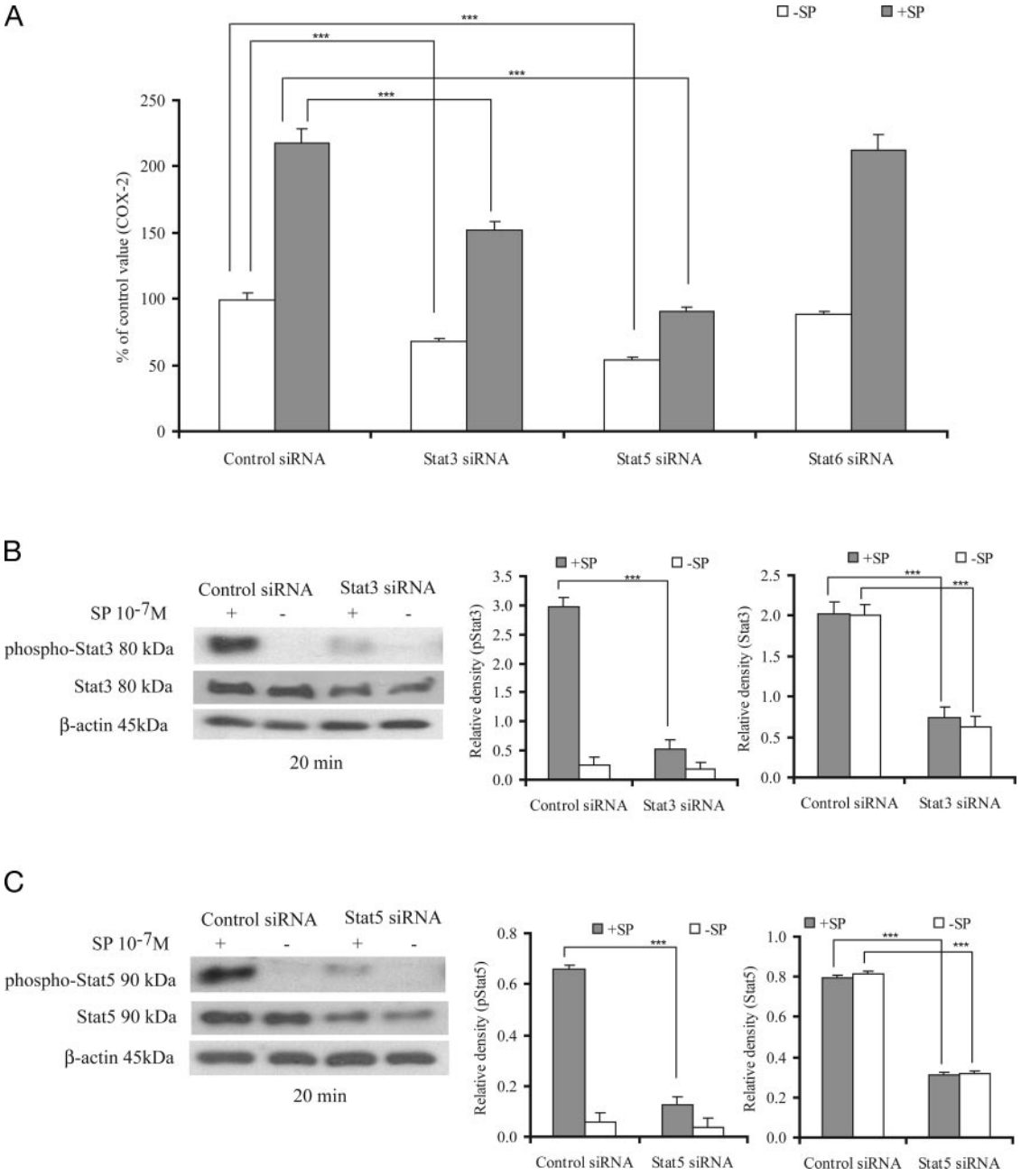

FIGURE 7. RNA interference of STAT3 and STAT5 inhibits COX-2 promoter transcriptional activity. *A*, NCM460-NK-1R cells were transiently transfected with siRNAs of STAT3, STAT5, or negative control together with the wild-type COX-2 promoter reporter plasmids. All cells were also transfected with a control pRL-TK plasmid for *Renilla* luciferase signal. Transfected cells were serum starved overnight and treated with SP (10^{-7} M) for 4 h. Cell extracts were prepared to measure COX-2 promoter activity. Data are expressed as means ± SEM (percentage of relative firefly luciferase activity, normalized to control group without SP stimulation, which was normalized as 100%) and are representative of six independent samples. *B*, Control siRNA- or STAT3 siRNA-transfected cells were lysed, and equal amount of proteins was used to determine the levels of STAT3, phospho-STAT3, and β-actin. Densitometric analyses of Western blot experiments with phospho-STAT3 and STAT3 indicated that STAT3 siRNA significantly inhibited STAT3 protein expression and SP-induced phospho-STAT3 signal. *C*, Control siRNA- or STAT5 siRNA-transfected cells were lysed, and equal amounts of proteins were used to determine the levels of STAT5, phospho-STAT5, and β-actin. Densitometric analyses of Western blot experiments with phospho-STAT5 and STAT5 indicated that STAT5 siRNA significantly inhibited STAT5 protein expression and SP-induced phospho-STAT5 signal. ***, Indicates $p < 0.001$ vs control group. Western blot results are representative of three independent experiments.

relevance of the SP-COX-2 in vitro response. Using both pharmacological inhibitors and siRNA approaches, we demonstrate that the signal transduction cascade following SP-NK-1R engagement activates the JAK2 and STAT3/5 pathways, leading to subsequent COX-2 expression and PGE_2 secretion. We also present molecular evidence that the sp3 and sp4 GAS motifs on the COX-2 promoter are important in the mediation of STAT-dependent COX-2 promoter activity in response to SP.

Our results from the site-directed mutations of the two GAS motifs (sp3 and sp4) indicate that the STAT binding sites on the COX-2 promoter appear to be the primary elements important for SP-induced COX-2 transcription (Fig. 8). This is primarily supported by our results depicted in Fig. 8 demonstrating complete inhibition of SP-induced COX-2 promoter activity when either the sp3 or the sp4 GAS motif is mutated. Yamaoka et al. (40) also showed that LPS-induced COX-2 gene regulation might involve

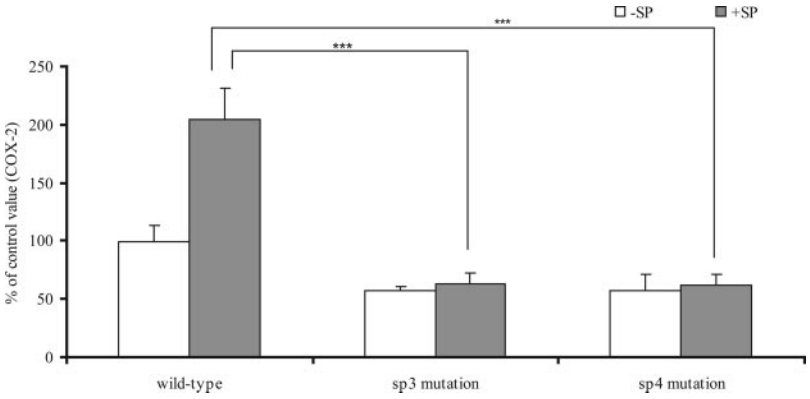

FIGURE 8. Both of STAT-binding GAS motifs on COX-2 promoter are essential for COX-2 promoter transcriptional activity. NCM460-NK-1R cells were transiently transfected with a wild-type COX-2 promoter construct or COX-2 promoter constructs with mutations on either the sp3 GAS site or the sp4 GAS site together with a control pRL-TK plasmid. All transfected cells were serum starved overnight and treated with SP (10^{-7} M) for 4 h. Cell extracts were used to measure wild-type/mutated COX-2 promoter luciferase activity. Data are expressed as means ± SEM (percentage of relative firefly luciferase activity, normalized to control group without SP stimulation, which was normalized as 100%) and are representative of six independent samples. ***, Indicates $p < 0.001$ vs control group.

STAT5 binding to sp3 and sp4 motifs on the COX-2 promoter. However, the COX- 2 promoter also contains several other elements for transcription factors that regulate its expression, including NF-κB and p38 and its downstream effector MSK1-CREB/ATF1. For example, the CRE element in the COX-2 promoter is important for *Clostridium difficile* toxin A-induced, p38-mediated COX-2 expression and PGE_2 secretion (46). Moreover, IL-1β and bradykinin B2 receptor-induced COX-2 expression is primarily mediated via NF-κB activation (47, 48). However, it appears unlikely that NF-κB and p38 are involved in SP-induced COX-2 gene expression because as we show in this study, pharmacologic inhibition of p38 or NF-κB did not affect SP-mediated PGE_2 production in NCM460-NK-1R colonocytes (Fig. 2*B*), although SP activates both NF-κB (13, 14) and p38 (data not shown) in these, and as well as in other cells (11, 41).

We demonstrate in this study that NK-1R coupling phosphorylates JAK2, STAT3, and STAT5 (Fig. 4), and these signaling molecules are involved in COX-2 expression and PGE_2 secretion in response to SP (Figs. 5–7). To our knowledge, the association of the NK-1R to JAK and STAT signaling pathways has not been previously recognized. We also present evidence that a NK-1R-mediated, PKC-dependent signaling pathway upstream of JAK and STAT is involved in SP-associated JAK-STAT phosphorylation and COX-2 activation. Similarly, binding of thyrotropin to the G protein-coupled thyrotropin receptor activates STAT3 via a PKC-dependent pathway (49). Moreover, although SP induces phosphorylation of PKCδ, PKCθ, and PKCε (14), our results with specific inhibitors indicate that PKCθ, but not PKCδ and PKCε, is involved in SP-induced JAK2, STAT3, and STAT5 phosphorylation (Fig. 5*C*) and COX-2 expression (Fig. 5*D*). Together, our previous findings (14) and results presented in this current study indicate a major role for PKC activation in the cellular pathways activated by SP-NK-1R engagement, with PKCθ mediating activation of the JAK-STAT-COX-2 pathway, leading to release of PGE_2, while PKCδ mediates SP-induced NF-κB activation and transcription of proinflammatory genes.

Previous studies also indicate that the *src* family of kinases mediates phosphorylation of JAK and STAT (50). However, SP did not induce c-*src* phosphorylation in NCM460-NK-1R colonocytes (data not shown), confirming our prior observations in U-373 MG cells (15). Moreover, although EGFR has been found to mediate *src* kinase and STAT phosphorylation (51), and EGFR activation is linked to COX-2 activation and release of PGE_2 in colon cancer cells (52), our results with the EGFR inhibitor AG1478 indicate that EGFR activation does not affect SP-induced STAT phosphorylation (data not shown) and PGE_2 secretion (Fig. 2*B*).

Our results demonstrating the mechanism by which SP and its high-affinity receptor activate COX-2 gene expression and PGE_2 secretion in human colonocytes might be relevant to the pathophysiology of several colonic functions and pathologic conditions in which both SP/NK-1R interactions and PGs have suggested playing a functional role. For example, cholera toxin induces PGE_2 and fluid secretion that can be prevented by COX-2 inhibitors (53), as well as NK-1R (54) antagonists. Moreover, *C. difficile* toxin A stimulates intestinal COX-2 activation and PGE_2 release (46, 55), and induces expression of SP and NK-1R (5, 34). Most importantly, administration of either COX-2 inhibitors (46, 55) or NK-1R antagonists (6) substantially reduces inflammatory diarrhea in response to this toxin. COX-2 gene and PGE_2 were found to mediate healing of chronic colonic inflammation (56, 57), and studies with NK-1R-deficient mice indicated a similar protective role for this receptor in the pathophysiology of chronic colitis (17). Our current study further indicates that SP-induced PGE_2 release may represent another mechanism by which SP promotes resolution of colonic inflammation, a response mediated via a novel NK-1R-PKCθ-JAK2-STAT-COX-2 pathway.

Disclosures

The authors have no financial conflict of interest.

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