

Investigate small particles with unparalleled sensitivity
Amnis® CellStream® Flow Cytometry System

For Research Use Only. Not for use in diagnostic procedures.



Luminex®
complexity simplified.



Substance P Stimulates Cyclooxygenase-2 and Prostaglandin E₂ Expression through JAK-STAT Activation in Human Colonic Epithelial Cells

This information is current as of November 30, 2021.

Hon-Wai Koon, Dezheng Zhao, Yanai Zhan, Sang Hoon Rhee, Mary P. Moyer and Charalabos Pothoulakis

J Immunol 2006; 176:5050-5059; ;
doi: 10.4049/jimmunol.176.8.5050
<http://www.jimmunol.org/content/176/8/5050>

References This article **cites 56 articles**, 22 of which you can access for free at:
<http://www.jimmunol.org/content/176/8/5050.full#ref-list-1>

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2006 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Substance P Stimulates Cyclooxygenase-2 and Prostaglandin E₂ Expression through JAK-STAT Activation in Human Colonic Epithelial Cells¹

Hon-Wai Koon,* Dezheng Zhao,* Yanai Zhan,* Sang Hoon Rhee,* Mary P. Moyer,[†] and Charalabos Pothoulakis^{2*}

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. PGE₂ biosynthesis is controlled by cyclooxygenase-1 (COX-1) and COX-2, whose induction involves the STATs. In this study, we examined whether SP stimulates PGE₂ 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 PGE₂ production by 2 h. Pharmacologic experiments showed that PGE₂ production is dependent on newly synthesized COX-2, but COX-1 protein. Inhibition of protein kinase C θ (PKC θ), but not PKC ϵ and PKC δ , 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 PGE₂ production. Transient transfection with JAK2 short-interfering 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 PGE₂ 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- κ B (11, 12) and NF- κ B-regulated cytokines (13, 14). SP-NK-1R interactions also induce proliferation of colonic epithelial as well as non-epithelial 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 PGE₂ production (25), while a specific COX-2 inhibitor reduces spinal SP-evoked PGE₂ release (26). SP, at supraphysiologic concentrations (μ M), stimulates PGE₂ release from rat intrapulmonary bronchi and trachea preparations via NK-1R activation (27, 28). Moreover, release of PGE₂ is involved in SP-induced relaxation of rat trachea (28), and mouse tracheal preparations via activation of PGE₂ 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). PGE₂ 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.

*Gastrointestinal Neuropeptide Center, Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215; and [†]INCELL, San Antonio, TX 78249

Received for publication October 19, 2005. Accepted for publication January 31, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by Grant DK 47343 (to C.P.) from the National Institutes of Health and by a research fellowship award (to H.-W.K.) by the Crohn's and Colitis Foundation of America.

² Address correspondence and reprint requests to Dr. Charalabos Pothoulakis, Beth Israel Deaconess Medical Center, Gastrointestinal Neuropeptide Center, Division of Gastroenterology, Dana 501, 330 Brookline Avenue, Boston, MA 02215. E-mail address: cpothoul@bidmc.harvard.edu

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-interfering RNA; TFA, trifluoroacetic acid.

(32) reported that PGE₂ 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 up-regulated in several forms of intestinal inflammation (33–36), including on colonic epithelial cells (37, 38), we used nontransformed human colonic epithelial NCM460 cells overexpressing NK-1R to investigate whether SP activates COX-2 and PGE₂ 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 PGE₂ 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 PGE₂ 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 μ M), indomethacin (20 μ M), FR122047 (20 μ M), cycloheximide (50 μ M), JAK inhibitor I (10–40 μ M), SB203580 (20 μ M), AG1478 (2 μ M), rottlerin (mallotoxin 0.1–1 μ M), caffeic acid phenethyl ester (CAPE, 20 μ M), specific protein kinase C θ (PKC θ) pseudosubstrate peptide inhibitor (Myr-LHQRGAIKQA KVHHVKC-NH₂ 10 μ M), PKC ϵ pseudosubstrate peptide inhibitors (EAVSLKPT 10 μ M) (Calbiochem), and CJ-12,255 (0.1–1 μ M) (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 μ l 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 μ g/lane) were loaded for Western blot experiments.

PGE₂ ELISA

PGE₂ levels in conditioned medium were determined by ELISA kits (R&D Systems), according to the manufacturer's instructions. Results were expressed as mean \pm SEM (pg/ml).

Western blot analyses

SP-treated cells were lysed in $1 \times$ lysis buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.01% bromophenol 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 milliamperes 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 (Tyr⁷⁰¹), phospho-STAT3 (Tyr⁷⁰⁵), phospho-STAT5 (Tyr⁶⁹⁴), phospho-STAT6 (Tyr⁶⁴¹) (Cell Signaling Technology), phospho-JAK3 (Tyr⁹⁸⁰), 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-interfering 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

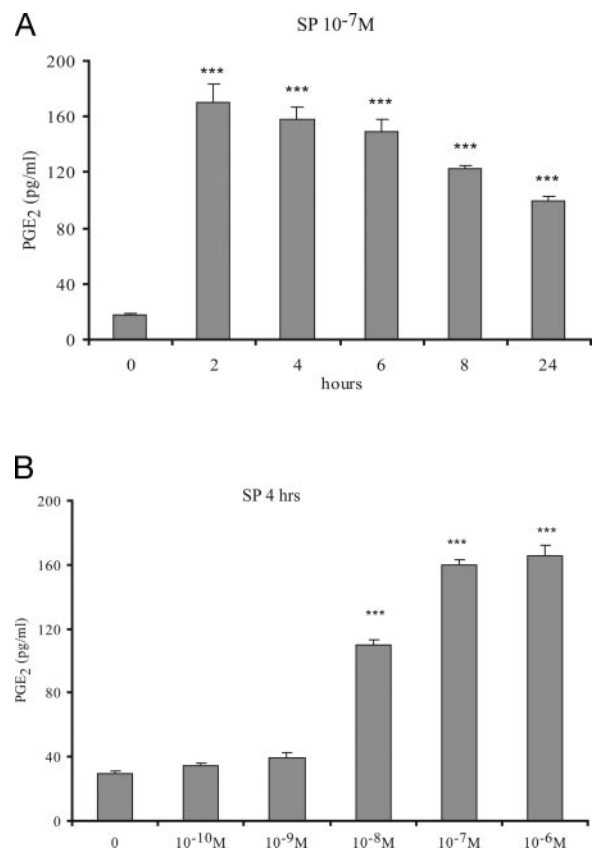


FIGURE 1. SP stimulates PGE₂ secretion in NCM460-NK-1R colonocytes expressing NK-1R. Serum-starved NCM460-NK-1R cells were treated with SP (10^{-7} M) for the indicated time points (A) or with different doses (0 – 10^{-6} M) of SP for 4 h (B). Conditioned medium was collected, and PGE₂ levels were measured by ELISA. SP induces significant PGE₂ release in time-dependent and dose-dependent manner. Data are expressed as mean \pm 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'-TCTCTCCCCAAAACAAG-3' (sp3) and 5'-ATTTCCCTGTTA 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 PGE₂ production in colonocytes in a time- and dose-dependent manner

To start investigating whether SP causes PGE₂ 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 PGE₂ ELISA. SP induced a rapid release of PGE₂ as early as 2 h after SP exposure, remained at high levels for 6 h (Fig. 1A), but slowly reduced at 8 and 24 h, albeit at higher levels than controls (Fig. 1A). The response of PGE₂ production within 4 h was dose dependent, with detectable SP induction at 10⁻⁸ M (Fig.

1B). In all subsequent experiments, we used a submaximal dose of SP (10⁻⁷ M).

SP-induced PGE₂ 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 PGE₂ production, NCM460-NK-1R cells were pretreated with the COX-2-specific inhibitor NS-398 (60 μ M), or the COX-1/2 inhibitor indomethacin (20 μ M), the COX-1-specific inhibitor FR122047 (20 μ M), or the NK-1R antagonist CJ-12,255 (1 μ M), 30 min before SP stimulation for 4 h. SP-induced PGE₂ was completely inhibited by NS-398, indomethacin, and CJ-12,255, but not FR122047 (Fig. 2A), indicating that PGE₂ 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 μ M) led to diminished SP-induced PGE₂ levels (Fig. 2B), 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 PGE₂ production (39, 40). To investigate whether this pathway is involved in NK-1R-associated PGE₂ secretion, NCM460-NK-1R cells were pretreated with the JAK inhibitor, JAK inhibitor I (40 μ M), for 30 min, followed by SP (10⁻⁷ M) stimulation for 4 h. Our results showed that JAK inhibitor I completely inhibited SP-induced PGE₂ production (Fig.

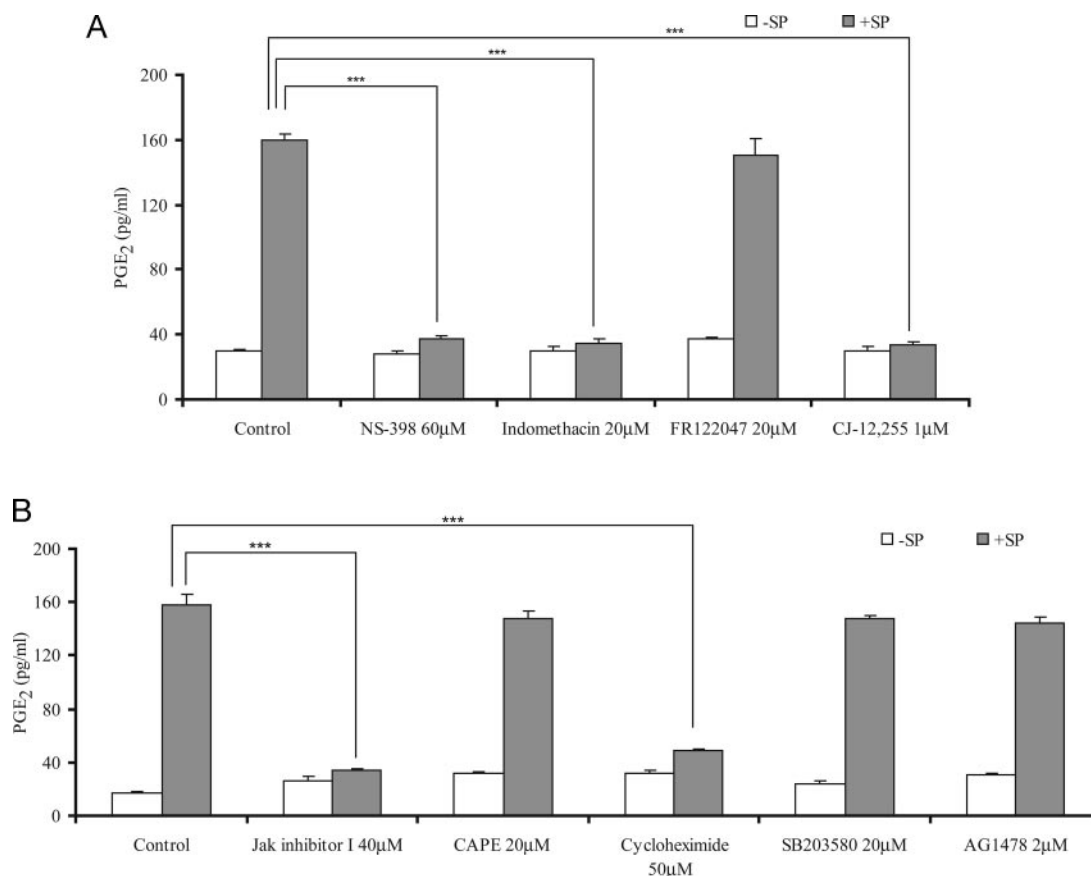


FIGURE 2. SP-induced PGE₂ secretion depends on JAK2 and COX-2, but not COX-1 expression. *A*, Serum-starved NCM460-NK-1R cells were pretreated with indomethacin (20 μ M), NS-398 (60 μ M), FR122047 (20 μ M), or CJ-12,255 (1 μ M) for 30 min, followed by TFA or SP (10⁻⁷ M) for 4 h. *B*, Serum-starved NCM460-NK-1R cells were pretreated with JAK inhibitor I (40 μ M), CAPE (20 μ M), cycloheximide (50 μ M), SB203580 (20 μ M), or AG1478 (2 μ M) for 30 min, followed by TFA or SP (10⁻⁷ M) exposure for 4 h. Conditioned cell medium was collected, and PGE₂ levels were measured by ELISA. PGE₂ release is dependent on de novo COX-2 protein synthesis and JAK pathways. COX-1 does not appear to mediate SP-induced PGE₂ synthesis. Data are expressed as means \pm SEM and are representative of six independent samples. ***, Indicates $p < 0.001$ vs control group.

2B), indicating the NK-1R-mediated JAK signaling is essential for SP-induced PGE₂ 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 PGE₂ secretion (Fig. 2B), indicating lack of involvement of these NK-1R-related signaling pathways in SP-induced PGE₂ synthesis.

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

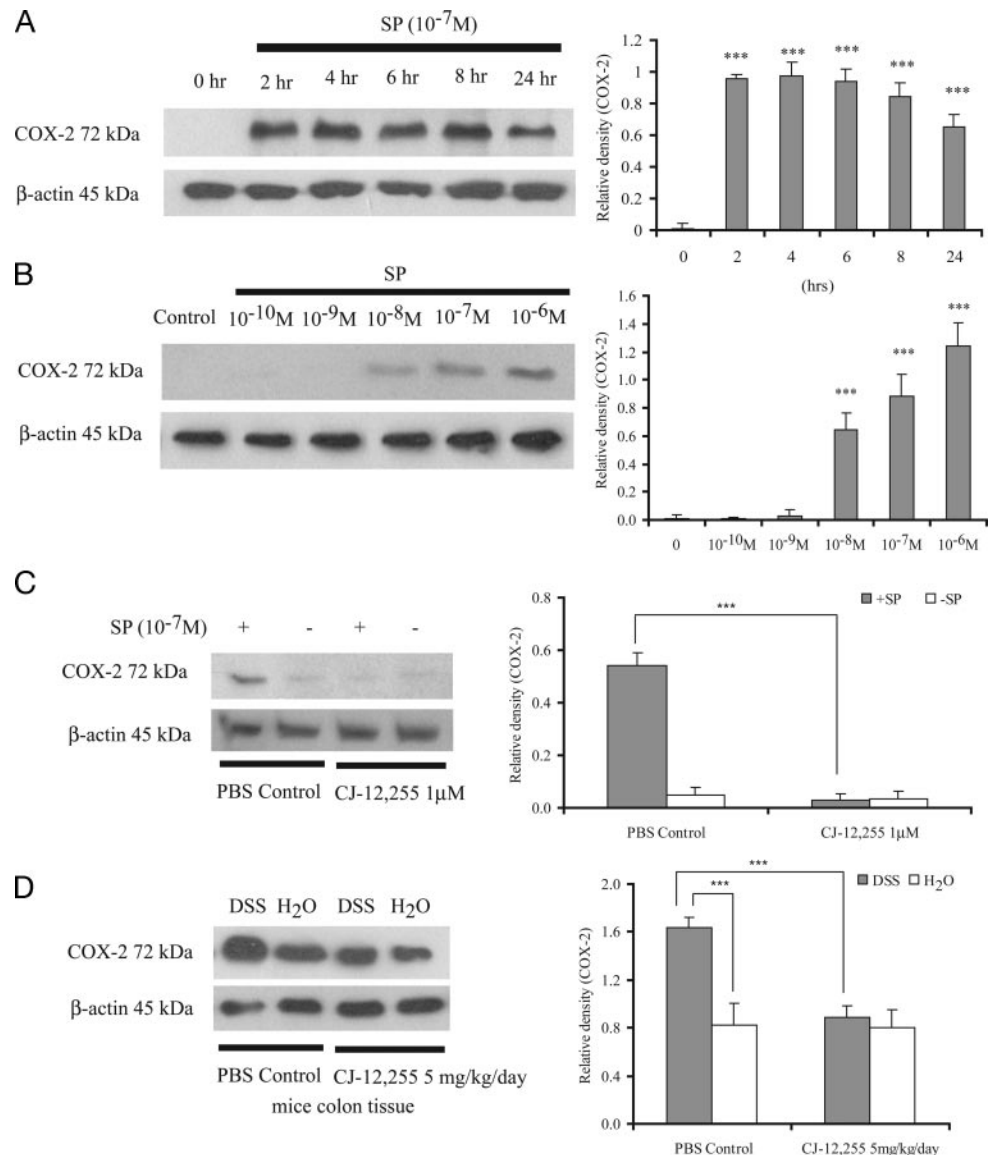
PGs, including PGE₂, are the enzymatic products of COX. Because our results indicated that COX-2, but not COX-1, mediates SP-induced PGE₂ secretion, we examined the expression of COX-2 by Western blotting. We found that COX-2 protein expression was increased as early as 2 h after SP exposure (Fig. 3A), remained elevated after 4–6 h, and then started to decline at 8 and 24 h ($p < 0.001$) (Fig. 3A). This SP-induced COX-2 time-dependent expression was correlated with the PGE₂ secretion pattern shown in Fig. 1A. SP also substantially increased COX-2 expres-

sion ($p < 0.001$) in a dose-dependent manner with detectable induction at 10⁻⁸ M, and higher induction at 10⁻⁷ and 10⁻⁶ M (Fig. 3B). 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. 2A, SP-induced PGE₂ 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. 3C). 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. 3D). However, administration of CJ-12,255 significantly reduced COX-2 levels to levels seen in control, non-DSS-exposed mice ($p < 0.001$; Fig. 3D).

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 H₂O 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. 3C, 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 PGE₂ 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. 4A). 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. 4B). 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. 2A 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. 4C).

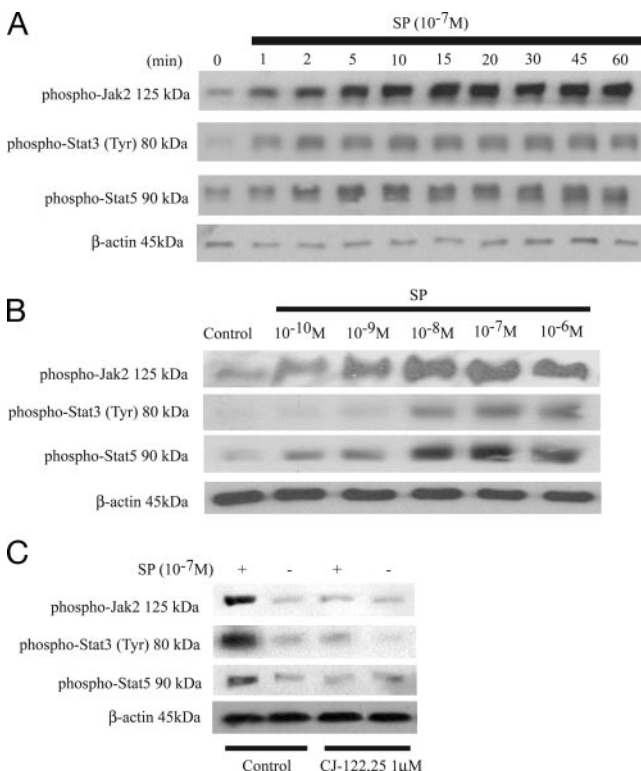


FIGURE 4. SP induces JAK2, STAT3, and STAT5 phosphorylation. Serum-starved NCM460-NK-1R cells were treated with SP (10⁻⁷ M) for the indicated time points (A) and with different doses (0–10⁻⁶ M) of SP for 20 min (B). Serum-starved NCM460-NK-1R cells were pretreated with NK-1R antagonist CJ-12,255 (1 μM) 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 fractionated 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. 5A), 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. 5B), 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. 5C) and COX-2 expression (*p* < 0.001) (Fig. 5D), 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 PGE₂ 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. 6A). 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. 6B). 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. 6A). Densitometric analysis of the results from Fig. 6B 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

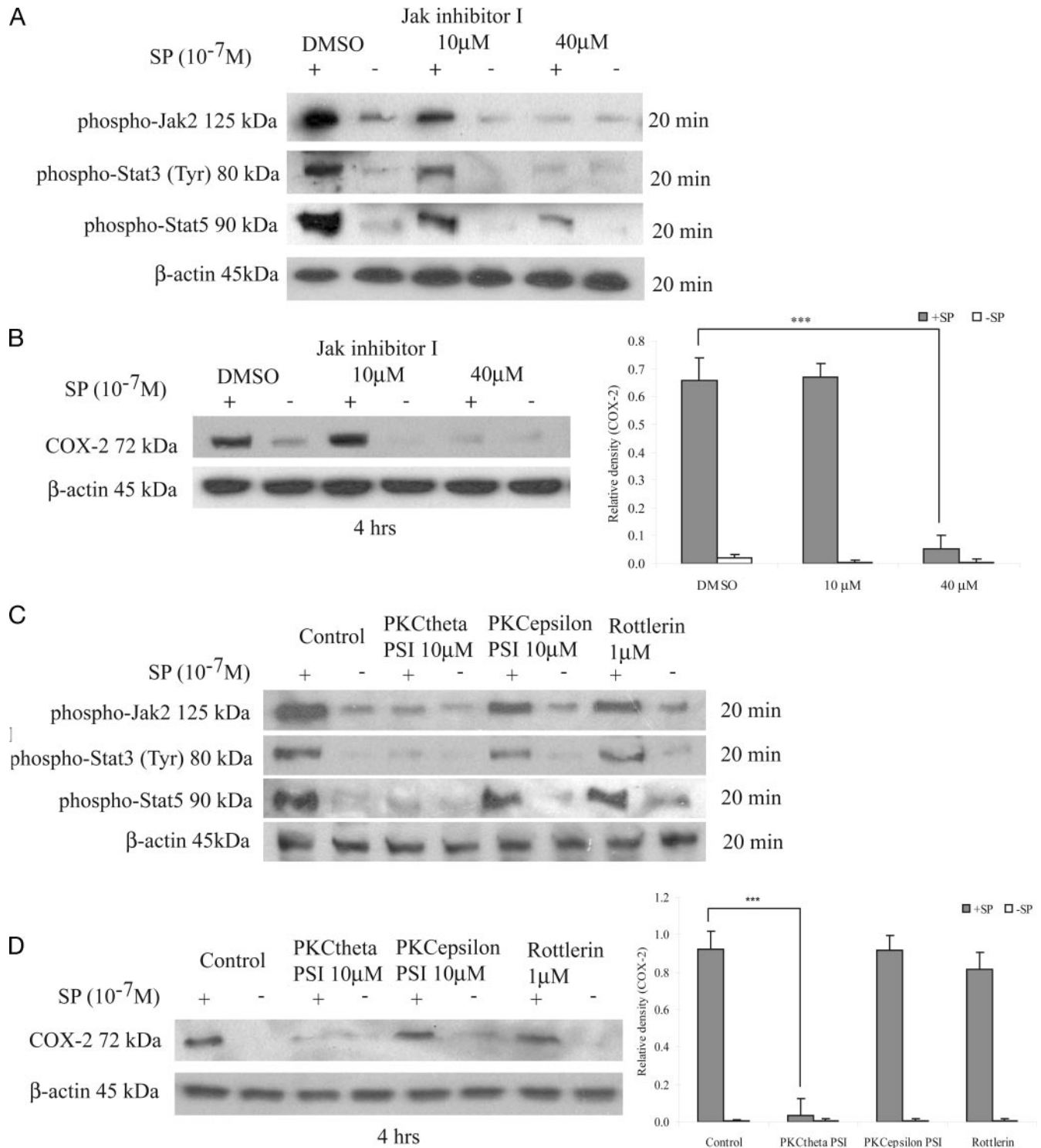
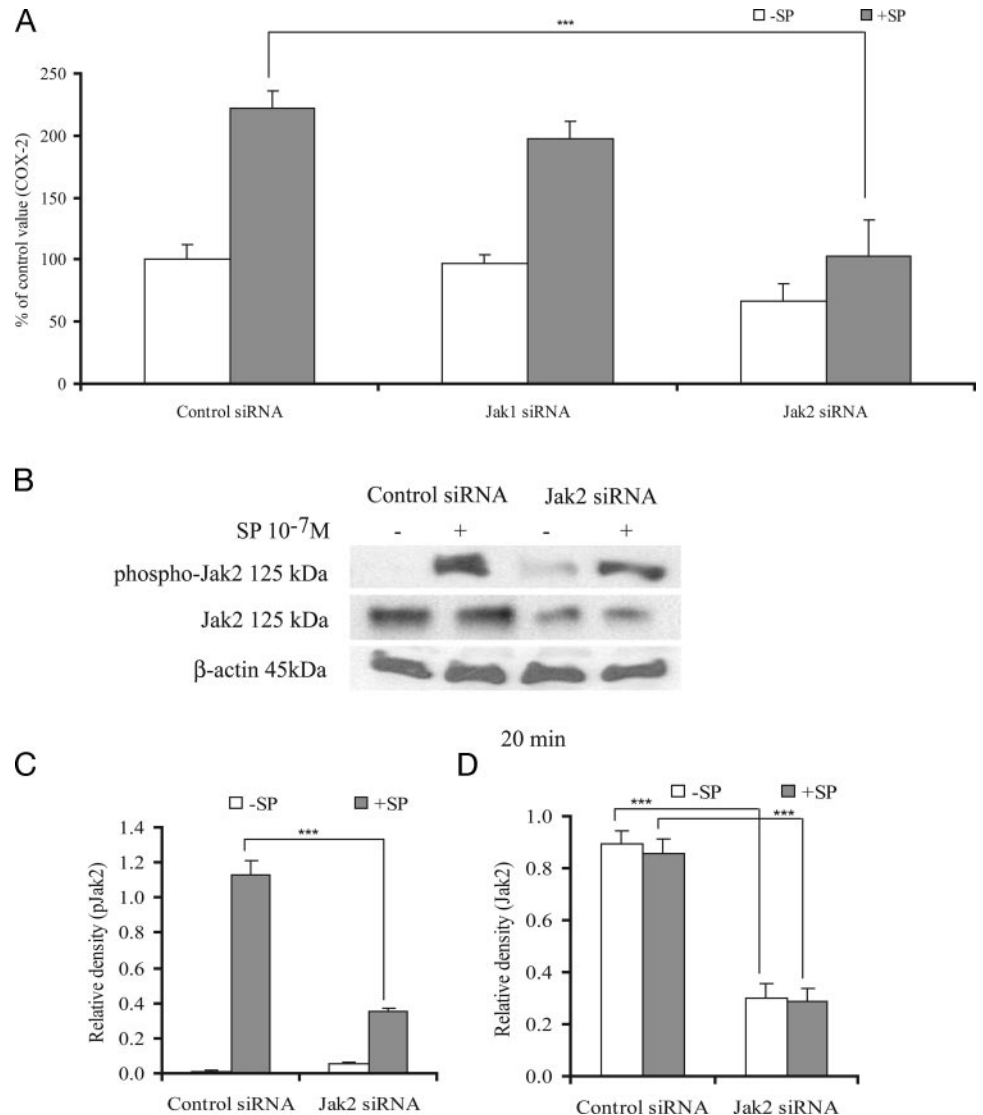


FIGURE 5. Pharmacological blockades of JAK and PKC θ inhibit SP-induced STAT3/5 phosphorylation and COX-2 expression. Serum-starved NCM460-NK-1R cells were pretreated with various doses of JAK inhibitor I or vehicle control DMSO for 30 min, followed by SP (10^{-7} M) exposure for 20 min (A) or 4 h (B). Serum-starved NCM460-NK-1R cells were pretreated with a PKC θ pseudosubstrate inhibitor (10 μ M), a PKC ϵ pseudosubstrate inhibitor (10 μ M), rottlerin (1 μ M), 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. 7A). STAT5

siRNA exerted a more profound effect on SP-induced COX-2 activity than STAT3 siRNA on (Fig. 7A). 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 \pm 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. 7B) and phospho-STAT5 expression as well as their endogenous protein expression (Fig. 7C). Densitometric analyses of the results (Fig. 7B) showed that STAT3 siRNA significantly reduced the SP-induced phospho-STAT3 signal by 83% (Fig. 7B) and STAT3 signal by \sim 63% (Fig. 7B). Moreover, incubation with STAT5 siRNA significantly inhibited SP-induced phospho-STAT5 signal by 80% (Fig. 7C) and nonphosphorylated STAT5 signal by \sim 61% (Fig. 7C). 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 PGE₂. 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. 3D), suggesting the pathophysiologic

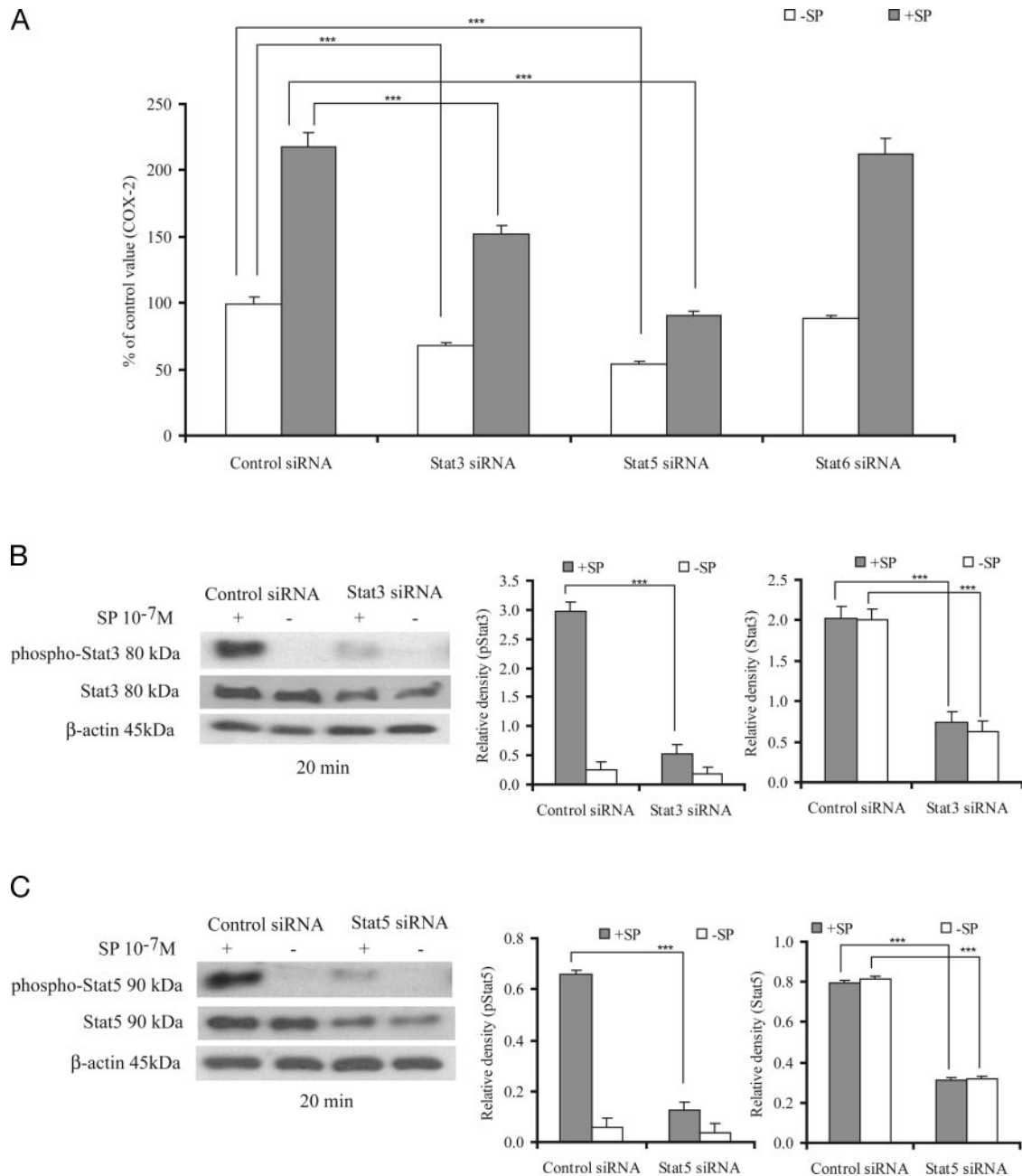
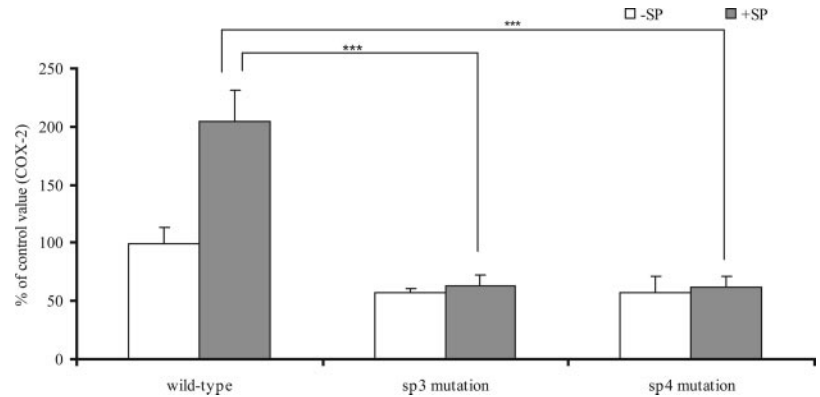


FIGURE 7. RNA interference of STAT3 and STAT5 inhibits COX-2 promoter transcriptional activity. **A**, NCM460-NK-1R cells were transiently transfected with siRNAs of STAT3, STAT5, or negative control together with the wild-type COX-2 promoter reporter plasmids. All cells were also transfected with a 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 \pm 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 PGE₂ 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 \pm 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- κ B 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 PGE₂ secretion (46). Moreover, IL-1 β and bradykinin B2 receptor-induced COX-2 expression is primarily mediated via NF- κ B activation (47, 48). However, it appears unlikely that NF- κ B and p38 are involved in SP-induced COX-2 gene expression because as we show in this study, pharmacologic inhibition of p38 or NF- κ B did not affect SP-mediated PGE₂ production in NCM460-NK-1R colonocytes (Fig. 2B), although SP activates both NF- κ B (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 PGE₂ 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 PKC δ , PKC θ , and PKC ϵ (14), our results with specific inhibitors indicate that PKC θ , but not PKC δ and PKC ϵ , is involved in SP-induced JAK2, STAT3, and STAT5 phosphorylation (Fig. 5C) and COX-2 expression (Fig. 5D). 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 PKC θ mediating activation of the JAK-STAT-COX-2 pathway, leading to release of PGE₂, while PKC δ mediates SP-induced NF- κ B 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 PGE₂ 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 PGE₂ secretion (Fig. 2B).

Our results demonstrating the mechanism by which SP and its high-affinity receptor activate COX-2 gene expression and PGE₂

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 PGE₂ 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 PGE₂ 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 PGE₂ 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 PGE₂ release may represent another mechanism by which SP promotes resolution of colonic inflammation, a response mediated via a novel NK-1R-PKC θ -JAK2-STAT-COX-2 pathway.

Disclosures

The authors have no financial conflict of interest.

References

- Chang, M. M., and S. E. Leeman. 1970. Isolation of a sialogic peptide from bovine hypothalamic tissue and its characterization as substance P. *J. Biol. Chem.* 245: 4784–4790.
- Holzer, P., and U. Holzer-Petsche. 1997. Tachykinins in the gut. I. Expression, release and motor function. *Pharmacol. Ther.* 73: 173–217.
- Maggi, C. A. 1990. Capsaicin-sensitive nerves in the gastrointestinal tract. *Arch. Int. Pharmacodyn. Ther.* 303: 157–166.
- Costa, M., J. B. Furness, R. Franco, I. Llewellyn-Smith, R. Murphy, and A. M. Beardsley. 1982. Substance P in nerve tissue in the gut. *Ciba Found. Symp.* 129–144.
- Castagliuolo, I., A. C. Keates, B. Qiu, C. P. Kelly, S. Nikulasson, S. E. Leeman, and C. Pothoulakis. 1997. Increased substance P responses in dorsal root ganglia and intestinal macrophages during *Clostridium difficile* toxin A enteritis in rats. *Proc. Natl. Acad. Sci. USA* 94: 4788–4793.
- Pothoulakis, C., I. Castagliuolo, J. T. LaMont, A. Jaffer, J. C. O'Keane, R. M. Snider, and S. E. Leeman. 1994. CP-96,345, a substance P antagonist, inhibits rat intestinal responses to *Clostridium difficile* toxin A but not cholera toxin. *Proc. Natl. Acad. Sci. USA* 91: 947–951.
- Hou, J. Y., M. F. Otterson, and S. K. Sarna. 1989. Local effect of substance P on colonic motor activity in different experimental states. *Am. J. Physiol.* 256: G997–G1004.
- Riegler, M., I. Castagliuolo, M. Wlk, and C. Pothoulakis. 1999. Substance P causes a chloride-dependent short-circuit current response in rabbit colonic mucosa in vitro. *Scand. J. Gastroenterol.* 34: 1203–1211.
- Pothoulakis, C., and J. T. Lamont. 2001. Microbes and microbial toxins: paradigms for microbial-mucosal interactions. II. The integrated response of the intestine to *Clostridium difficile* toxins. *Am. J. Physiol.* 280: G178–G183.
- Weinstock, J. V. 2004. The role of substance P, hemokinin and their receptor in governing mucosal inflammation and granulomatous responses. *Front. Biosci.* 9: 1936–1943.
- Lieb, K., B. L. Fiebich, M. Berger, J. Bauer, and K. Schulze-Osthoff. 1997. The neuropeptide substance P activates transcription factor NF- κ B and κ B-dependent gene expression in human astrocytoma cells. *J. Immunol.* 159: 4952–4958.

12. Simeonidis, S., I. Castagliuolo, A. Pan, J. Liu, C. C. Wang, A. Mykoniatis, A. Pasha, L. Valenick, S. Sougioultzis, D. Zhao, and C. Pothoulakis. 2003. Regulation of the NK-1 receptor gene expression in human macrophage cells via an NF- κ B site on its promoter. *Proc. Natl. Acad. Sci. USA* 100: 2957–2962.
13. Zhao, D., S. Kuhn-Moore, H. Zeng, A. Pan, J. S. Wu, S. Simeonidis, M. P. Moyer, and C. Pothoulakis. 2002. Substance P-stimulated interleukin-8 expression in human colonic epithelial cells involves Rho family small GTPases. *Biochem. J.* 368: 665–672.
14. Koon, H. W., D. Zhao, Y. Zhan, S. Simeonidis, M. P. Moyer, and C. Pothoulakis. 2005. Substance P-stimulated interleukin-8 expression in human colonic epithelial cells involves PKC δ activation. *J. Pharmacol. Exp. Ther.* 314: 1393–1400.
15. Castagliuolo, I., L. Valenick, J. Liu, and C. Pothoulakis. 2000. Epidermal growth factor receptor transactivation mediates substance P-induced mitogenic responses in U-373 MG cells. *J. Biol. Chem.* 275: 26545–26550.
16. Koon, H. W., D. Zhao, X. Na, M. P. Moyer, and C. Pothoulakis. 2004. Metalloproteinases and transforming growth factor- α mediate substance P-induced mitogen-activated protein kinase activation and proliferation in human colonocytes. *J. Biol. Chem.* 279: 45519–45527.
17. Castagliuolo, I., O. Morteau, A. C. Keates, L. Valenick, C. C. Wang, J. Zacks, B. Lu, N. P. Gerard, and C. Pothoulakis. 2002. Protective effects of neurokinin-1 receptor during colitis in mice: role of the epidermal growth factor receptor. *Br. J. Pharmacol.* 136: 271–279.
18. Wallace, J. L. 2001. Prostaglandin biology in inflammatory bowel disease. *Gastroenterol. Clin. North Am.* 30: 971–980.
19. Williams, C. S., M. Mann, and R. N. DuBois. 1999. The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene* 18: 7908–7916.
20. Perini, R. F., L. Ma, and J. L. Wallace. 2003. Mucosal repair and COX-2 inhibition. *Curr. Pharm. Des.* 9: 2207–2211.
21. Smith, W. L., D. L. DeWitt, and R. M. Garavito. 2000. Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.* 69: 145–182.
22. Morita, I. 2002. Distinct functions of COX-1 and COX-2. *Prostaglandins Other Lipid Mediat.* 68–69: 165–175.
23. Takeuchi, K., A. Tanaka, K. Suzuki, and H. Mizoguchi. 2001. Gastrointestinal sparing anti-inflammatory drugs: effects on ulcerogenic and healing responses. *Curr. Pharm. Des.* 7: 49–69.
24. Schwab, J. M., H. J. Schluesener, R. Meyermann, and C. N. Serhan. 2003. COX-3 the enzyme and the concept: steps towards highly specialized pathways and precision therapeutics? *Prostaglandins Leukotrienes Essent. Fatty Acids* 69: 339–343.
25. Rasley, A., I. Marriott, C. R. Halberstadt, K. L. Bost, and J. Anguita. 2004. Substance P augments *Borrelia burgdorferi*-induced prostaglandin E₂ production by murine microglia. *J. Immunol.* 172: 5707–5713.
26. Yaksh, T. L., D. M. Dirig, C. M. Conway, C. Svensson, Z. D. Luo, and P. C. Isakson. 2001. The acute antihyperalgesic action of nonsteroidal, anti-inflammatory drugs and release of spinal prostaglandin E₂ is mediated by the inhibition of constitutive spinal cyclooxygenase-2 (COX-2) but not COX-1. *J. Neurosci.* 21: 5847–5853.
27. Szarek, J. L., B. Spurlock, C. A. Gruetter, and S. Lemke. 1998. Substance P and capsacin release prostaglandin E₂ from rat intrapulmonary bronchi. *Am. J. Physiol.* 275: L1006–L1012.
28. Devillier, P., G. M. Acker, C. Advenier, J. Marsac, D. Regoli, and N. Frossard. 1992. Activation of an epithelial neurokinin NK-1 receptor induces relaxation of rat trachea through release of prostaglandin E₂. *J. Pharmacol. Exp. Ther.* 263: 767–772.
29. Fortner, C. N., R. M. Breyer, and R. J. Paul. 2001. EP2 receptors mediate airway relaxation to substance P, ATP, and PGE₂. *Am. J. Physiol.* 281: L469–L474.
30. Riegler, M., I. Castagliuolo, P. T. So, M. Lotz, C. Wang, M. Wik, T. Sogukoglu, E. Cosentini, G. Bischof, G. Hamilton, et al. 1999. Effects of substance P on human colonic mucosa in vitro. *Am. J. Physiol.* 276: G1473–G1483.
31. Manning, B. P., K. A. Sharkey, and G. M. Mawe. 2002. Effects of PGE₂ in guinea pig colonic myenteric ganglia. *Am. J. Physiol.* 283: G1388–G1397.
32. Hosoda, Y., S. Karaki, Y. Shimoda, and A. Kuwahara. 2002. Substance P-evoked Cl⁻ secretion in guinea pig distal colonic epithelia: interaction with PGE₂. *Am. J. Physiol.* 283: G347–G356.
33. Mantyh, C. R., S. R. Vigna, R. R. Bollinger, P. W. Mantyh, J. E. Maggio, and T. N. Pappas. 1995. Differential expression of substance P receptors in patients with Crohn's disease and ulcerative colitis. *Gastroenterology* 109: 850–860.
34. Pothoulakis, C., I. Castagliuolo, S. E. Leeman, C. C. Wang, H. Li, B. J. Hoffman, and E. Mezey. 1998. Substance P receptor expression in intestinal epithelium in *Clostridium difficile* toxin A enteritis in rats. *Am. J. Physiol.* 275: G68–G75.
35. Bhatia, M., A. K. Saluja, B. Hofbauer, J. L. Frossard, H. S. Lee, I. Castagliuolo, C. C. Wang, N. Gerard, C. Pothoulakis, and M. L. Steer. 1998. Role of substance P and the neurokinin 1 receptor in acute pancreatitis and pancreatitis-associated lung injury. *Proc. Natl. Acad. Sci. USA* 95: 4760–4765.
36. Blum, A. M., A. Metwali, C. Crawford, J. Li, K. Qadir, D. E. Elliott, and J. V. Weinstock. 2001. Interleukin 12 and antigen independently induce substance P receptor expression in T cells in murine schistosomiasis mansoni. *FASEB J.* 15: 950–957.
37. Stucchi, A. F., K. O. Shebani, S. E. Leeman, C. C. Wang, K. L. Reed, A. B. Fruin, A. C. Gower, J. P. McClung, C. D. Andry, M. J. O'Brien, et al. 2003. A neurokinin 1 receptor antagonist reduces an ongoing ileal pouch inflammation and the response to a subsequent inflammatory stimulus. *Am. J. Physiol.* 285: G1259–G1267.
38. Renzi, D., B. Pellegrini, F. Tonelli, C. Surrenti, and A. Calabro. 2000. Substance P (neurokinin-1) and neurokinin A (neurokinin-2) receptor gene and protein expression in the healthy and inflamed human intestine. *Am. J. Pathol.* 157: 1511–1522.
39. Chun, K. S., and Y. J. Surh. 2004. Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. *Biochem. Pharmacol.* 68: 1089–1100.
40. Yamaoka, K., T. Otsuka, H. Niuro, Y. Arinobu, Y. Niho, N. Hamasaki, and K. Izuhara. 1998. Activation of STAT5 by lipopolysaccharide through granulocyte-macrophage colony-stimulating factor production in human monocytes. *J. Immunol.* 160: 838–845.
41. Fiebich, B. L., S. Schleicher, R. D. Butcher, A. Craig, and K. Lieb. 2000. The neuropeptide substance P activates p38 mitogen-activated protein kinase resulting in IL-6 expression independently from NF- κ B. *J. Immunol.* 165: 5606–5611.
42. Martin, A. R., I. Villegas, and C. Alarcon de la Lastra. 2005. The COX-2 inhibitor, rofecoxib, ameliorates dextran sulphate sodium induced colitis in mice. *Inflamm. Res.* 54: 145–151.
43. Bolli, R., B. Dawn, and Y. T. Xuan. 2003. Role of the JAK-STAT pathway in protection against myocardial ischemia/reperfusion injury. *Trends Cardiovasc. Med.* 13: 72–79.
44. Kovanen, P. E., I. Juntila, K. Takaluoma, P. Saharinen, L. Valmu, W. Li, and O. Silvennoinen. 2000. Regulation of Jak2 tyrosine kinase by protein kinase C during macrophage differentiation of IL-3-dependent myeloid progenitor cells. *Blood* 95: 1626–1632.
45. Chang, Y. J., M. J. Holtzman, and C. C. Chen. 2004. Differential role of Janus family kinases (JAKs) in interferon- γ -induced lung epithelial ICAM-1 expression: involving protein interactions between JAKs, phospholipase C γ , c-Src, and STAT1. *Mol. Pharmacol.* 65: 589–598.
46. Kim, H., S. H. Rhee, E. Kokkotou, X. Na, T. Savidge, M. P. Moyer, C. Pothoulakis, and J. T. LaMont. 2005. *Clostridium difficile* toxin A regulates inducible cyclooxygenase-2 and prostaglandin E₂ synthesis in colonocytes via reactive oxygen species and activation of p38 MAPK. *J. Biol. Chem.* 280: 21237–21245.
47. Liu, W., N. Reinmuth, O. Stoeltzing, A. A. Parikh, C. Tellez, S. Williams, Y. D. Jung, F. Fan, A. Takeda, M. Akagi, et al. 2003. Cyclooxygenase-2 is up-regulated by interleukin-1 β in human colorectal cancer cells via multiple signaling pathways. *Cancer Res.* 63: 3632–3636.
48. Chen, B. C., C. C. Yu, H. C. Lei, M. S. Chang, M. J. Hsu, C. L. Huang, M. C. Chen, J. R. Sheu, T. F. Chen, T. L. Chen, et al. 2004. Bradykinin B2 receptor mediates NF- κ B activation and cyclooxygenase-2 expression via the Ras/Raf-1/ERK pathway in human airway epithelial cells. *J. Immunol.* 173: 5219–5228.
49. Park, Y. J., E. S. Park, M. S. Kim, T. Y. Kim, H. S. Lee, S. Lee, I. S. Jang, M. Shong, D. J. Park, and B. Y. Cho. 2002. Involvement of the protein kinase C pathway in thyrotropin-induced STAT3 activation in FRTL-5 thyroid cells. *Mol. Cell. Endocrinol.* 194: 77–84.
50. Silva, B. M. 2004. Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. *Oncogene* 23: 8017–8023.
51. Olayioye, M. A., I. Beuvink, K. Horsch, J. M. Daly, and N. E. Hynes. 1999. ErbB receptor-induced activation of STAT transcription factors is mediated by Src tyrosine kinases. *J. Biol. Chem.* 274: 17209–17218.
52. Coffey, R. J., C. J. Hawkey, L. Damstrup, R. Graves-Deal, V. C. Daniel, P. J. Dempsey, R. Chinery, S. C. Kirkland, R. N. DuBois, T. L. Jetton, and J. D. Morrow. 1997. Epidermal growth factor receptor activation induces nuclear targeting of cyclooxygenase-2, basolateral release of prostaglandins, and mitogenesis in polarizing colon cancer cells. *Proc. Natl. Acad. Sci. USA* 94: 657–662.
53. Beubler, E., R. Schuligoi, A. K. Chopra, D. A. Ribardo, and B. A. Peskar. 2001. Cholera toxin induces prostaglandin synthesis via post-transcriptional activation of cyclooxygenase-2 in the rat jejunum. *J. Pharmacol. Exp. Ther.* 297: 940–945.
54. Turvill, J. L., P. Connor, and M. J. Farthing. 2000. Neurokinin 1 and 2 receptors mediate cholera toxin secretion in rat jejunum. *Gastroenterology* 119: 1037–1044.
55. Alcantara, C., W. F. Stenson, T. S. Steiner, and R. L. Guerrant. 2001. Role of inducible cyclooxygenase and prostaglandins in *Clostridium difficile* toxin A-induced secretion and inflammation in an animal model. *J. Infect. Dis.* 184: 648–652.
56. Reuter, B. K., S. Asfaha, A. Buret, K. A. Sharkey, and J. L. Wallace. 1996. Exacerbation of inflammation-associated colonic injury in rat through inhibition of cyclooxygenase-2. *J. Clin. Invest.* 98: 2076–2085.
57. Nitta, M., I. Hirata, K. Toshina, M. Murano, K. Maemura, N. Hamamoto, S. Sasaki, H. Yamauchi, and K. Katsu. 2002. Expression of the EP4 prostaglandin E₂ receptor subtype with rat dextran sodium sulphate colitis: colitis suppression by a selective agonist, ONO-AE1-329. *Scand. J. Immunol.* 56: 66–75.