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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 Pothoulakis²*

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 Cθ (PKCθ), but not PKCe 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 PGE₂ production. Transient transfection with JAK2 short-interfering RNA reduced 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.

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*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 colonicocytes has not been examined, and the COX isof orm(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 NCM-460 cells overexpressing NK-1R to investigate whether SP activates COX-2 and PGE2 secretion in colonicocytes 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 colonicocytes. 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 M3 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 × 10⁵ cells/plate) overnight in M3 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), FR228047 (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-LHQRRAIKQA KVHHYVC-NH2 10 μM), PKCe 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 asphyxiation prior to harvest. Necropsy was performed under aseptic conditions.

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 ± SEM (pg/ml).

Western blot analyses

SP-treated cells were lysed in 1X 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 (Tyr1022/1023), phospho-JAK2 (Tyr1007/1008), phospho-STAT1 (Tyr701), phospho-STAT3 (Tyr705), phospho-STAT5 (Y845), phospho-STAT5 (Y694), phospho-STAT6 (Y641) (Cell Signaling Technology), phospho-JAK3 (Y606), 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 × 10⁵ 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.
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 TCTCTGTTGAAACAAG-3′ from −850 to −831), were modified to 5′-TCTCTCCCCAAACAAACAAG-3′ (sp3) and 5′-ATTTCCTGTGA 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 isof orm is involved in SP-induced PGE2 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 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. Pre-treatment of colonocytes with the protein synthesis inhibitor cycloheximide (50 μM) 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 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 PGE2 production (Fig. 2B).
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 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. 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 and H2O to induce colitis, with/without CJ-12,255 (5 mg/kg/day) p.o. 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, A and B, 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 (0–10⁻⁸ 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 PKCδ-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 PKCe (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 PKCe, 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 PKCe, 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...
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 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.
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 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.

**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
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...
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-kB 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-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 PGE2 production in NCM460-NK-1R colonocytes (Fig. 2B), 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 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 thylotropin 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. 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 PKCθ mediating activation of the JAK-STAT-COX-2 pathway, leading to release of PGE2, 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 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 pathophysiologic of several colonic functions and pathologic conditions in which both SP/NK-1R interactions and PGs have suggested playing a functional role. For example, cholela 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 pathophysiologic 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-PKCθ-JAK2-STAT-COX-2 pathway.

Disclosures
The authors have no financial conflict of interest.

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