Proteinase-activated Receptor-2 Induces Cyclooxygenase-2 Expression through β-Catenin and Cyclic AMP-response Element-binding Protein*

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Hongying Wang†‡, Shoubin Wen‡, Nigel W. Bunnett†, Richard Leduc‡, Morley D. Hollenberg§, and Wallace K. MacNaughton**‡

From the †Inflammation Research Network and the Departments of **Physiology and Biophysics and †Pharmacology and Therapeutics, University of Calgary, Calgary, Alberta T2N 4N1, Canada, the ‡Department of Surgery and Physiology, University of California, San Francisco, California 94143-0104, and the §University of Sherbrooke, Sherbrooke, Quebec J1K 2R1, Canada

Chronic inflammation of mucosae is associated with an increased cancer risk. Tumorigenesis in these tissues is associated with the activity of some proteinases, cyclooxygenase-2 (COX-2), and β-catenin. Serine proteinases participate in both inflammation and tumorigenesis through the activation of proteinase-activated receptor-2 (PAR2), which up-regulates COX-2 by an unknown mechanism. We sought to determine whether β-catenin participated in PAR2-induced COX-2 expression and through what cellular mechanism. In A549 epithelial cells, we showed that PAR2 activation increased COX-2 expression through the β-catenin/T cell factor transcription pathway. This effect was dependent upon ERK1/2 MAPK, which inhibited the β-catenin-regulating protein, glycogen synthase kinase-3β, and induced the activity of the cAMP-response element-binding protein (CREB). Knockdown of CREB by small interfering RNA revealed that PAR2-induced β-catenin transcriptional activity and COX-2 expression were CREB-dependent. A co-immunoprecipitation assay revealed a physical interaction between CREB and β-catenin. Thus, PAR2 up-regulated COX-2 expression via an ERK1/2-mediated activation of the β-catenin/Tcf-4 and CREB pathways. These findings reveal new cellular mechanisms by which serine proteinases may participate in tumor development and are particularly relevant to cancers associated with chronic mucosal inflammation, where serine proteinases are abundant and COX-2 overexpression is a common feature.

Patients with chronic inflammatory diseases of mucosae, including those of the airway and intestine, have an increased risk for the development of cancer. Serine proteinases have been implicated as key factors in mucosal inflammation and the formation and metastasis of tumors. These proteinases trigger specific cellular responses through proteinase-activated receptors (PARs), G-protein-coupled receptors that are activated by proteolytic cleavage of the extracellular N terminus at a specific amino acid sequence, revealing a new N-terminal “tethered ligand” that binds to and activates the receptor (1, 2). PAR2, one of the four members of this receptor family, can be activated by trypsin (3), tryptase (4), and the tumor-derived proteinase matriptase (5) to stimulate processes ranging from inflammation and pain perception to tumorigenesis (3, 7–9). Tumor cells, especially those of epithelial origin, express a high level of PAR2 (10, 11). Tryptsin and matriptase are commonly overexpressed in tumor cells and in their microenvironment at concentrations compatible with PAR2 activation (12, 13).

We have shown previously that the activation of PAR2 stimulates COX-2 expression (14). COX-2 is expressed early in carcinogenesis and very likely plays a role in the development of cancer (15–17), as it correlates with tumor invasion and poor clinical outcome (18). The mechanisms by which PAR2 activation induces the expression of COX-2 are still unclear. However, it is known that the cox2 gene can be induced by the transcriptional activity of β-catenin. Many cancers are characterized by mutations of either β-catenin or components of its degradation complex, such that it accumulates in the cytoplasm, translocates to the nucleus, and binds to the nuclear binding protein Tcf-4 to induce the expression of genes associated with cell proliferation and tumorigenesis, including c-myc (19), cyclin D1 (20), and cox2 (21, 22).

We hypothesized that the β-catenin transcription pathway is involved in PAR2-induced COX-2 expression. Here, using a human epithelial cancer cell line, we show that a selective PAR2 agonist and endogenous activators of PAR2 increase the expression of COX-2 through the activation of the β-catenin/Tcf-4 signaling pathway and an ERK-dependent pathway. Moreover, PAR2 induced the activation of CREB, which directly interacted with β-catenin in the process of PAR2-induced COX-2 expression.

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1 Recipient of a post-doctoral award from the Canadian Institutes of Health Research, the Canadian Association of Gastroenterology, and Axcan Pharma.

2 Senior Scholar of the Alberta Heritage Foundation for Medical Research. To whom correspondence should be addressed: Dept. of Physiology and Biophysics, University of Calgary, 3330 Hospital Dr. NW, Calgary, Alberta T2N 4N1, Canada. Tel.: 403-220-5882; Fax: 403-283-3840; E-mail: wmacnaug@ucalgary.ca.

3 The abbreviations used are: PAR, proteinase-activated receptor; AP, activating peptide; CRE, cyclic AMP-response element; CREB, cyclic AMP-response element-binding protein; COX, cyclooxygenase; MEK, mitogen-activated protein kinase; EGFr, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GSK, glycogen synthase kinase; siRNA, small interfering RNA; PGE2, prostaglandin E2; TBE, Tcf binding element; RT, reverse transcription; Tcf, T cell factor; MAPK, mitogen-activated protein kinase.
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EXPERIMENTAL PROCEDURES

Cell Culture—The human airway epithelial cell line A549 (ATCC, Manassas, VA) was grown in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (Sigma) supplemented with 10% fetal bovine serum (Invitrogen). Cells were serum-starved for 1 h prior to stimulation with PAR2-AP, trypsin, or matriptase.

Chemicals and Reagents—The inhibitors for MEK, PD98059 and U0126, were purchased from Promega (Nepean, Ontario, Canada). The inhibitors for EGFr tyrosine kinase (AG1478) and Src (PP1) were from Calbiochem. Trypsin was from Sigma, and actinomycin D was from Invitrogen. SLIGRL-NH2 and LRGILS-NH2 were prepared at the peptide synthesis facility of the University of Calgary. The composition and purity of the peptides and the concentrations of stock solutions were verified by using high pressure liquid chromatography, mass spectrometry, and amino acid analysis. Matriptase was prepared essentially as described previously (23).

Plasmids—TOPFLASH and FOPFLASH (24) were generously provided by Drs. M. C. Hung and J. Deng at the M. D. Anderson Cancer Center, Houston, TX. Wild-type and mutant κB-luciferase reporters were generously provided by Drs. B. Winston and Y. Huang at the University of Calgary. pTK-RL was purchased from Promega.

Immunoblot—Whole cell lysates and cytosolic extracts were prepared as described previously (25). Proteins were separated by SDS-PAGE. The antibodies used and their suppliers were as follows: anti-COX-2 (Cayman Chemical, Ann Arbor, MI); anti-phospho-ERK1/2, anti-total ERK1/2, and anti-phospho-GSK-3α/β (Ser-21/9) (Cell Signaling Technology Inc., Danvers, MA); anti-total β-catenin and anti-total GSK-3β (BD Transduction Laboratories); anti-phospho-CREB (Ser-133) (R&D Systems, Minneapolis, MN); anti-total CREB and anti-actin (Sigma); and anti-active β-catenin and anti-Tcf-4 (Upstate, Temecula, CA). Anti-mouse and anti-rabbit IgGs conjugated to peroxidase (The Jackson Laboratory, Bar Harbor, ME) were used as secondary antibodies. Immunoblots were developed by enhanced chemiluminescence (ECL, Amersham Biosciences). All the membranes were rebotted for actin, which was used as a loading control. The intensity of the bands was quantified using Quantity One™ software (Bio-Rad). Densitometry data were expressed as the ratio to actin.

Enzyme Immunoassay of PGE2—For PGE2 assay, A549 cells were seeded onto 12-well plates. After treatment for 3 h, the media were collected and diluted 10 times, and the amount of PGE2 in the diluted samples was determined using an enzyme immunoassay kit according to the manufacturer’s instructions (Cayman Chemical). The amount of PGE2 formed and released in response to stimulation was calculated according to the standard curve established on the same plate.

RT-PCR—Total RNA was extracted using the RNeasy mini-kit (Qiagen, Valencia, CA). The RT reaction was performed using 500 ng of total RNA that was reverse-transcribed into cDNA using a random hexamer primer (Invitrogen). PCR was performed with a HotStarTaq® master mix kit (Qiagen) according to the manufacturer’s instructions. PCR for actin was done as an internal control. The annealing temperatures for COX-2 and actin were 65 and 50 °C, respectively. Primer sequences for COX-2 were as follows: sense, 5’-TTCAAT-GAGATTGTGGAAAATGC-3’; and antisense, 5’-AGAT-CATCTCTGCTGTAGTATTTT-3’. PCR products were then separated in a 2% agarose gel with ethidium bromide.

Transient Transfection and Luciferase Assay—Transient transfection was performed with β-catenin-driven luciferase (TOPFLASH) or κB-driven luciferase plasmid as a reporter for transcriptional activity. The transfection agent Lipofectamine™ (Invitrogen) was incubated with DNA in serum-free media for 30 min before being added to cells and incubating for an additional 2 h. Cell lysates for luciferase activity were collected 24 h after transfection, and cells were treated with PAR2-AP for 3 h before harvesting. Plasmids with mutant Tcf-4 sites (FOPFLASH) or with mutant κB binding sites (mutant κB-luciferase) were used as controls in the transfection assays. All data were normalized by pTK-RL. The luciferase assays were performed with a Dual-Luciferase assay kit (Promega).

Nuclear Fractionation and Co-immunoprecipitation—After treatment of the cells, nuclear fractionation and co-immunoprecipitation were performed as described previously (26). Briefly, the cells were suspended in hypotonic buffer (10 mM HEPES with 10 mM KCl, 0.1 mM EDTA, and 0.1 mM EGTA) for 15 min to allow the cells to swell. Nonidet P-40 was then added to the cell suspension, and the mixture was vortexed vigorously for 10 s. The homogenate was centrifuged (10,000 × g) at 4 °C for 30 s. The nuclear pellet was resuspended in 25 μl of ice-cold nuclear extraction buffer (20 mM HEPES with 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol). After incubation on ice for 30 min, the samples were centrifuged, and the supernatant was collected as the nuclear fraction.

Anti-Tcf-4 or anti-CREB antibody (2 μg) with 40 μl of protein A/G-PLUS-agarose beads (Santa Cruz Biotechnology) was added to 200 μg of nuclear extract in the buffer mentioned above and incubated at 4 °C overnight. The beads were washed three times with this buffer, and the proteins were dissolved and boiled in sample buffer for SDS-PAGE. After transfer, the membranes were blotted for β-catenin and Tcf-4. The nuclear extract without immunoprecipitation was blotted with anti-CREB antibody and used as a loading control for the CREB pulldown assay. Negative controls were conducted in a similar manner, but with either mouse IgG or rabbit serum instead of specific antibody during immunoprecipitation.

siRNA for β-Catenin and CREB—siRNA was performed to knock down targeted genes. Duplex oligonucleotide siRNA was purchased from Dharmacon (Lafayette, CO). The target sequences of siRNA oligonucleotides were as follows: β-catenin, 5’-AAGUCCUGUAUGAGUGGGAAC-3’; CREB, 5’-GCTCGAGAGTGTCGTAGAA-3’; and a nonspecific duplex oligonucleotide control (CONTROL™, Dharmacon). siRNA (400 pmol/well in a 6-well plate) was transfected using 10 μl of Lipofectamine™. Treatment of cells with PAR2-AP occurred 24 h after transfection.

Immunocytochemistry—A549 cells were seeded onto 8-well chamber slides. After treatment with or without PAR2-AP for 3 h, cells were fixed in methanol for 30 min at -20 °C. After washing, cells were blocked with 10% bovine serum albumin in
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**RESULTS**

**Activation of PAR$_2$ Increases COX-2 Expression**—We used the human lung carcinoma-derived A549 epithelial cell line as a model for PAR$_2$-induced COX-2 expression (14). We showed by immunoblotting that the selective PAR$_2$-activating peptide, SLIGRL-NH$_2$, but not the reverse-sequence inactive peptide, LRGILS-NH$_2$, induced a significant increase in COX-2 protein expression (Fig. 1A) 3 h after PAR$_2$ activation. The endogenous phosphate-buffered saline for at least 2 h. The cells were then incubated with anti-$\beta$-catenin antibody (1:100 in 2% bovine serum albumin) or anti-active $\beta$-catenin antibody (1:50 in 2% bovine serum albumin) overnight at 4°C. Fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Invitrogen) was applied at 1:150 for a 2-h incubation. Nuclei were stained with 4',6-diamidino-2-phenyldole (10 $\mu$g/ml) for 5 min. Imaging was performed with an Olympus FluoView 1000 confocal laser scanning microscope.

**COX-2 Promoter Mutations**—The wild-type full-length COX-2 promoter-luciferase construct was provided by Dr. M. C. Hung. Mutations in the TBE site and the CRE site in the promoter were made by PCR-based site-directed mutagenesis (27). The CRE site (−1079/−1073) was mutated from CTTTTGAT to CTTTGGGC. The CRE site (−60/−56) was mutated from CGTCA to GAGCT. A double mutation was built by inserting the TBE mutation fragment into a HindIII-Neol-digested CRE-mutated luciferase vector. Clones were verified by restriction digests and sequencing.

**Statistical Analysis**—Data are presented as the means ± S.E. Comparison of more than two groups was made using analysis of variance with a post hoc Tukey test. Comparison of two groups was made using Student's $t$ test for unpaired data. An associated probability ($p$) value of $<0.05$ was considered significant.

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**FIGURE 1. Activation of PAR$_2$-induced COX-2 expression.** A, cell lysates were collected for immunoblot after treatment with HEPES (Con), reverse peptide (RP), PAR$_2$-AP (25 or 50 $\mu$m), trypsin (10 $\mu$m), or matriptase (Matr; 50 $\mu$m) for 3 h. The level of PGE$_2$ in the supernatant was measured by enzyme immunoassay. B, the level of $\beta$-catenin during the immunoprecipitation. After treatment for 3 h, the cells were collected for luciferase assay. The data were normalized to DMSO, dimethyl sulfoxide. *, $p < 0.05$ compared with the control group ($n \geq 3$).

**FIGURE 2. $\beta$-Catenin mediates PAR$_2$-induced COX-2 expression.** A, A549 cells were transfected with TOPFLASH (0.5 $\mu$m) or FOPFLASH (0.5 $\mu$m) and pTK-RL (0.005 $\mu$m) 1 day before the treatment. After treatment for 3 h, the cells were collected for luciferase assay. The data were normalized to Renilla luciferase activity. B, after treatment with HEPES (Con), PAR$_2$-AP (AP), trypsin (Try), or matriptase (Matr), the nuclear extract (NE) was isolated and used for co-immunoprecipitation (IP) of Tcf-4. The level of $\beta$-catenin was measured by Western blotting and normalized to Tcf-4 levels. Negative control (Neg) represents immunoblotting performed with mouse IgG during the immunoprecipitation. C, cells were transfected with CONTROL RNA (CRNA) or siRNA against $\beta$-catenin (SiRNA-$\beta$-catenin) with TOPFLASH or FOPFLASH and pTK-RL. One day after transfection, the cells were treated with PAR$_2$-AP for 3 h with subsequent processing for luciferase reporter assay. Data are expressed as a percentage of PAR$_2$-AP-treated control RNA cells. D, cytosolic extracts and whole cell lysates were prepared for Western blotting to measure $\beta$-catenin, COX-2, and actin, respectively. E, cells were transfected with kbp-luciferase (0.5 $\mu$m) or mutant kbp-luciferase (0.5 $\mu$m) and pTK-RL (0.005 $\mu$m) 1 day before the treatment. After treatment for 3 h, the cells were collected for luciferase assay. The data were normalized to Renilla luciferase activity. TNA$\alpha$, tumor necrosis factor-$\alpha$. *, $p < 0.05$ compared with control group ($n \geq 3$).
activators of PAR$_2$, trypsin and matriptase, also stimulated COX-2 protein expression (Fig. 1A). Furthermore, PAR$_2$ activation by PAR$_2$-AP, trypsin, and matriptase increased PGE$_2$ secretion at this time point (Fig. 1B), indicating that PAR$_2$ activation induced the expression of functional COX-2.

RT-PCR showed that the up-regulation of COX-2 enzyme triggered by PAR$_2$-AP, trypsin, and matriptase correlated with an increase in COX-2 mRNA (Fig. 1C). Pretreatment with the inhibitor of transcription, actinomycin D, completely abolished the PAR$_2$-AP-induced increase in COX-2 mRNA (Fig. 1D), indicating that PAR$_2$ induced COX-2 expression at the transcriptional level.

**β-Catenin/Tcf-4 Pathway Mediates PAR$_2$-induced COX-2 Expression**—Activation of PAR$_2$ significantly elevated β-catenin transcriptional activity as demonstrated by the TOPFLASH luciferase reporter assay (Fig. 2A). When Tcf-4 was immunoprecipitated from nuclear extracts of A549 cells exposed to PAR$_2$-AP, trypsin, or matriptase, we observed a significant increase of coprecipitated β-catenin compared with control cells (Fig. 2B), indicating that PAR$_2$ activation increased the binding of β-catenin to Tcf-4 in the nucleus. To test whether the activation of β-catenin was required for PAR$_2$-induced COX-2 expression, we used siRNA to selectively knock down β-catenin. Transfection of A549 cells with β-catenin-targeted siRNA substantially reduced the transcriptional activity (Fig. 2C) and protein level (Fig. 2D) of β-catenin and significantly attenuated PAR$_2$-induced COX-2 expression (Fig. 2D).

Because NF-κB is also important for transcriptional regulation of COX-2, we tested whether NF-κB was activated by PAR$_2$ signaling by using a κB-driven luciferase reporter. Treatment with tumor necrosis factor-α was used as a positive control for the activation of the NF-κB pathway (Fig. 2E). PAR$_2$-AP at 25 and 50 μM did not significantly change NF-κB activity. However, when the concentration of PAR$_2$-AP was increased to 100 μM, a concentration above that which activated β-catenin transcriptional activity, we did observe an elevation in κB-luciferase activity (Fig. 2E).

PAR$_2$ Activation of β-Catenin Occurs through an ERK-dependent Pathway—The activation of PAR$_2$ by activating peptide (PAR$_2$-AP) or trypsin stimulated the ERK/1/2 MAPK pathway as determined by an increase in phosphorylated ERK1/2 (Fig. 3A). Inhibitors of MEK (PD98059 and U0126), the kinase that activates ERK1/2, significantly blocked PAR$_2$-induced β-catenin transcriptional activity (Fig. 3B) and reduced the binding of β-catenin to Tcf-4 (Fig. 3C). In addition, the expression of COX-2 induced by PAR$_2$-AP and trypsin was reduced by the inhibition of ERK1/2 activation (Fig. 3D). These data indicated that ERK1/2 activation induced by PAR$_2$ led to β-catenin activation and COX-2 expression.

**Inactivation of GSK-3β by an ERK-dependent Pathway**—GSK-3β is part of the degradation complex that targets β-catenin to the proteasome. Phosphorylation of GSK-3β at Ser-9 inhibits its activity, and β-catenin transcriptional activity subsequently increases. To test whether ERK1/2 induces β-catenin expression through inactivation of GSK-3β by phosphorylation of Ser-9, we measured the inactivated (phosphorylated Ser-9) form of GSK-3β by immunoblot analysis and found a significant accumulation of inactive GSK-3β (Fig. 4A). Surprisingly, there was no significant change in the total amount of β-catenin (data not shown) or in the nuclear level of β-catenin (Fig. 2B, lanes 1 and 2). However, although total β-catenin did not change, we demonstrated an increase in the N-terminally dephosphorylated, active form of β-catenin after PAR$_2$ activation (Fig. 4A). Significantly, the MEK inhibitor U0126 dramatically blocked the PAR$_2$-stimulated accumulation of both the inactivated phospho-GSK-3β and active N-terminally dephosphorylated β-catenin (Fig. 4B). These results strongly suggested that PAR$_2$ caused an ERK1/2-mediated inhibition of GSK-3β concurrent with the accumulation of N-terminally dephosphorylated, active β-catenin that could translocate to the nucleus to induce COX-2 expression (Fig. 2B). The nuclear accumulation of the active form of β-catenin was demonstrated by immunocytochemistry. The primarily membrane distribution of inactive β-catenin was not affected by exposure of cells to SLIGRL-NH$_2$ (Fig. 4C). However, PAR$_2$ activation resulted in a
substantial increase in the nuclear localization of active β-catenin immunoreactivity (Fig. 4C).

EGF receptor Is Not Involved in PAR2-induced β-Catenin Activation—EGF receptor has been shown to increase β-catenin nuclear translocation and transcriptional activity (28), and PAR2 has been shown to transactivate EGF receptor through Src (29, 30). To test whether the activated EGF receptor might account for PAR2-induced β-catenin activation, we used inhibitors of EGF receptor tyrosine kinase activity (AG1478) and Src (PP1). Neither of these inhibitors blocked PAR2-induced β-catenin activation (Fig. 3B).

CREB Mediates PAR2-induced COX-2 Expression—CREB is another transcription factor that participates in COX-2 expression. Thus, we tested whether CREB was also involved in PAR2-induced COX-2 expression. We found that PAR2 activation by activating peptide or trypsin triggered CREB phosphorylation at Ser-133 (Fig. 5B), significantly reduced this interaction (Fig. 6B, lanes 1–4). Moreover, immunoprecipitation of CREB, followed by immunoblotting for β-catenin, showed that PAR2-AP, trypsin, and matriptase all substantially enhanced the binding of β-catenin to CREB compared with the control group (Fig. 6B, lanes 1–4). Moreover, the MEK inhibitors significantly reduced this interaction (Fig. 6B, lanes 5 and 6), suggesting that CREB not only physically, but also functionally, interacted with β-catenin. Promoter analysis showed that the PAR2 activating peptide dramatically increased the activity of the full-length wild-type COX-2 promoter-driven luciferase reporter (Fig. 6D). Mutation of either the TBE site (T-M, Fig. 6C) or the CRE site (C-M, Fig. 6C) in the COX-2 promoter significantly blocked the PAR2-induced COX-2 promoter activation (Fig. 6D). The mutation of both sites (TC-M, Fig. 6C) did not further reduce COX-2 activation (Fig. 6D). These results suggest there was a crosstalk between β-catenin and CREB and that both are required for PAR2-induced COX-2 expression.

DISCUSSION

Tumorigenesis is a complex, multifactorial process in which proteinases, β-catenin, and COX-2 have been implicated. In the present study, we investigated the effects of two serine proteinases, matriptase and trypsin, both of which signal through PAR2 and have been implicated in tumorigenesis. Our data provide a clear link between these serine proteinases, PAR2, and the β-catenin transcription pathway in the stimulation of COX-2 expression. We found that activation of PAR2 caused an increase in functional COX-2 in the A549 tumor cell line via a mechanism involving β-catenin, the ERK1/2 MAPK, and the interaction of TBE- and CREB-mediated transcription.

COX-2 is induced by inflammatory and mitogenic stimuli and is highly expressed in colorectal, gastric, lung, and breast carcinomas. Selective inhibitors of COX-2 are effective in the prevention and treatment of some cancers (31–35). However, the mechanism by which the level of COX-2 is enhanced in cancer is not well established. We have previously shown that activation of PAR2 can stimulate the expression of COX-2 in A549 cells through a mechanism involving cytosolic phospholipase A2, increased intracellular calcium, ERK1/2, and Src-mediated EGF receptor transactivation (14). Here we show that the activation of ERK1/2 is required for subsequent deactivation of GSK-3β and activation of β-catenin transcriptional activity to induce COX-2 expression. It has been shown that ERK-
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FIGURE 5. CREB is involved in PAR2-induced COX-2 expression. A, after pretreatment with the vehicle (dimethyl sulfoxide (DMSO)) or the inhibitors of MEK (PD98059 (PD) and U0126), A549 cells were challenged with PAR2-AP or trypsin for 20 min. Cell lysates were then collected for measurement of phospho-CREB (Ser-133) (P-CREB) and total CREB by Western blotting. Con, control; B, cells were transfected with CREB siRNA or CONTROL™ RNA (C-RNA). Whole cell lysates were collected to measure CREB and actin by Western blotting. C, cells were transfected with CREB siRNA or CONTROL™ RNA (C-RNA) followed by PAR2-AP treatment. Whole cell lysates were collected to measure COX-2 and actin by Western blotting. Densitometry revealed that knockdown of CREB resulted in a significant reduction in COX-2 expression. *, p < 0.05 compared with the control RNA group (C-RNA) (n ≥ 3).

FIGURE 6. Interaction of β-catenin and CREB is required for PAR2-induced COX-2 expression. A, A549 cells were transfected with siRNA against CREB or with CONTROL™ RNA (cRNA) combined with TOPFLASH and pTK-RL. One day after transfection, the cells were treated with PAR2-AP for 3 h, and the cell lysates were collected for luciferase assay. *, p < 0.05 compared with the control RNA group not treated with PAR2-AP; #, p < 0.05 compared with the PAR2-AP-treated control RNA group (n ≥ 3). B, after treatment, nuclear fractions were collected, and immunoprecipitation (IP) was performed with anti-CREB antibody. β-Catenin was visualized by Western blotting. Negative control (Neg) represents blotting conducted without antibody during the immunoprecipitation. A whole cell extract (WE) was loaded as a positive control. The nuclear fraction without immunoprecipitation was used and blotted for β-catenin and CREB is required for PAR2-induced COX-2 expression. The EGFr-independent component involves β-catenin.

Recent findings have demonstrated the importance of the N-terminally unphosphorylated or “active” form of β-catenin in the activation of the β-catenin/Tcf-4 pathway (43, 44). In the present study, we demonstrated that PAR2 activation significantly increased the level of the active form of β-catenin in the nucleus, which is comparable with the previously observed 2-fold increase induced by Wnt-1 (43). PAR2-induced activation of the β-catenin/Tcf-4 pathway without accumulation of total β-catenin is consistent with previous findings that accumulation of the active form does not require ongoing synthesis of β-catenin (45) and that accumulation of total β-catenin is insufficient (43, 44) or does not account (46) for β-catenin-dependent signaling.
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CREB, which can be activated by Ser-133 phosphorylation in response to several signaling pathways (47), specifically binds to CRE to regulate transcription of numerous genes (48). We showed that ERK1/2 is required for the activation of CREB. In addition, CREB physically interacts with β-catenin, and this interaction is required for β-catenin to activate COX-2 promoter activity. The crosstalk between β-catenin and CREB, indicated by the co-immunoprecipitation data, is in keeping with a previous study demonstrating a role for the CREB site in β-catenin-dependent transcriptional activation of WISP-1, a Wnt-induced secreted protein (49). A CREB/β-catenin interaction has also been documented for gastrin-mediated up-regulation of cyclin D1 (6). Compared with single mutations, double mutations of TBE and CRE sites in the COX-2 promoter did not further reduce PAR2-induced COX-2 transcriptional activation. This observation suggested that β-catenin and CREB used the same mechanism to up-regulate COX-2 expression. However, the precise mechanism by which β-catenin and CREB interact with CRE and Tcf-4/TBE to promote COX-2 up-regulation appears quite complex and remains an important topic for further study.

One of the implications of this work is that the proteinase-PAR2 signaling pathway we have described may represent a valid therapeutic target for cancer therapy. Specifically, inhibition of PAR2 may block tumorigenic epithelial cell signaling pathways. Unfortunately, despite considerable effort, no suitable PAR2 antagonists are yet available. However, by inhibiting concurrently serine proteinase activity, β-catenin, and COX-2, the growth and metastasis of tumor cells could potentially be attenuated. This triple-target strategy merits consideration in the future.

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