

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

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Hongying Wang^{†1}, Shoubin Wen[‡], Nigel W. Bunnett[§], Richard Leduc[¶], Morley D. Hollenberg^{‡||}, and Wallace K. MacNaughton^{†***2}

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-3 β , 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),³ 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). Trypsin 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 Axcen 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, cAMP-response element; CREB, cAMP-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.

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, PD98058 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 κ B-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-3 α/β (Ser-21/9) (Cell Signaling Technology Inc., Danvers, MA); anti-total β -catenin and anti-total GSK-3 β (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 reblotted for actin, which was used as a loading control. The intensity of the bands was analyzed by 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'-TTCAAATGAGATTGTGGGAAAATTGC-3'; and antisense, 5'-AGATCATCTCTGCCTGAGTATCTT-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 κ B-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 κ B binding sites (mutant κ B-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 \times g) at 4 °C for 30 s. The nuclear pellet was resuspended in 25 μ l of ice-cold nuclear extraction buffer (20 mM HEPES with 0.4 M 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 μ g) with 40 μ l of protein A/G-PLUS-agarose beads (Santa Cruz Biotechnology) was added to 200 μ g 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'-GCTCGAGAGTGTCTAGTAA-3'; and a nonspecific duplex oligonucleotide control (CONTROL™, Dharmacon). siRNA (400 pmol/well in a 6-well plate) was transfected using 10 μ l 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

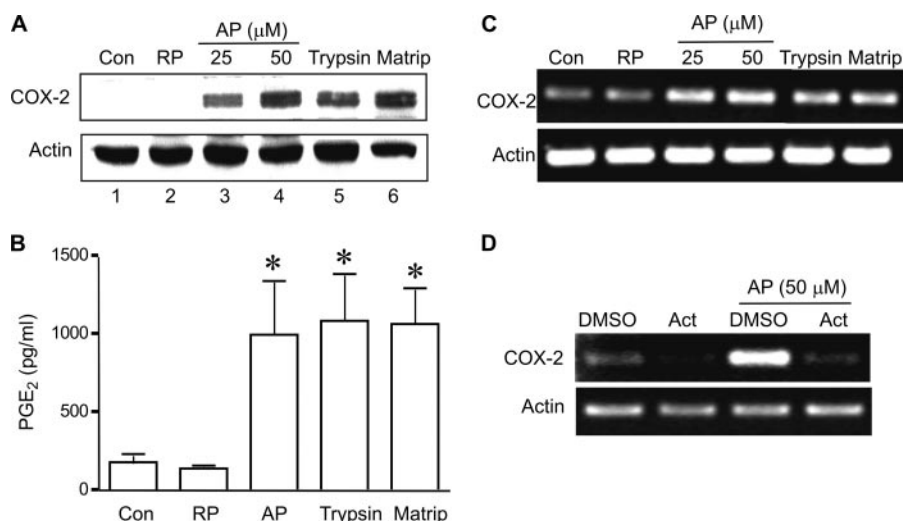


FIGURE 1. Activation of PAR₂-induced COX-2 expression. A, cell lysates were collected for immunoblot after treatment with HEPES (Con), reverse peptide (RP), PAR₂-AP (25 or 50 μM), trypsin (10 nM), or matriptase (Matrip; 50 nM) for 3 h. B, the level of PGE₂ in the supernatant was measured by enzyme immunoassay. C, cells were treated with PAR₂-AP, trypsin, or matriptase for 3 h, and the level of COX-2 mRNA was detected by RT-PCR. D, cells were pre-treated with actinomycin D (Act; 2 μg/ml) for 1 h before challenge with PAR₂-AP for 3 h. The level of COX-2 mRNA was then measured by RT-PCR. DMSO, dimethyl sulfoxide. *, $p < 0.05$ compared with the control group ($n \geq 3$).

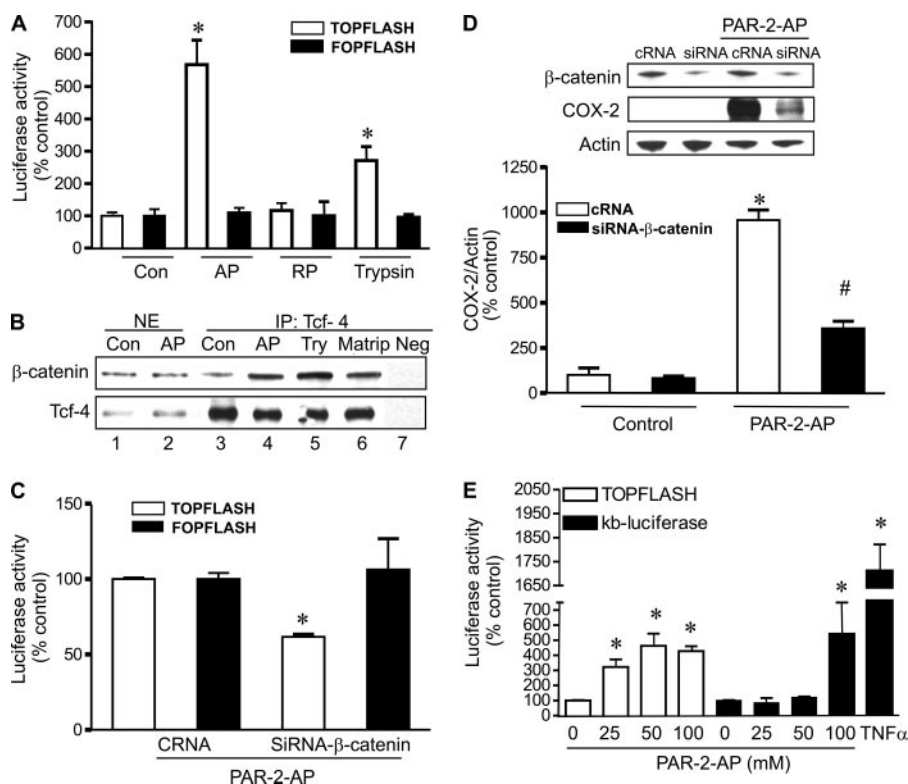


FIGURE 2. β -Catenin mediates PAR₂-induced COX-2 expression. A, A549 cells were transfected with TOPFLASH (0.5 μg) or FOPFLASH (0.5 μg) and pTK-RL (0.005 μg) 1 day before the treatment. After treatment for 3 h, the cells were collected for luciferase assay. The data were normalized to Renilla luciferase activity. B, after treatment with HEPES (Con), PAR₂-AP (AP), trypsin (Try), or matriptase (Matrip), the nuclear extract (NE) was isolated and used for co-immunoprecipitation (IP) of Tcf-4. The level of β -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 CONTROLTM RNA (CRNA) or siRNA against β -catenin (siRNA- β -catenin) with TOPFLASH or FOPFLASH and pTK-RL. One day after transfection, the cells were treated with PAR₂-AP for 3 h with subsequent processing for luciferase reporter assay. Data are expressed as a percentage of PAR₂-AP-treated control RNA cells. D, cytosolic extracts and whole cell lysates were prepared for Western blotting to measure β -catenin, COX-2, and actin, respectively. E, cells were transfected with κ B-luciferase (0.5 μg) or mutant κ B-luciferase (0.5 μg) and pTK-RL (0.005 μg) 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. TNF α , tumor necrosis factor- α . *, $p < 0.05$ compared with control group ($n \geq 3$).

phosphate-buffered saline for at least 2 h. The cells were then incubated with anti- β -catenin antibody (1:100 in 2% bovine serum albumin) or anti-active β -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-phenylindole (10 μg/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 TBE site (−1079/−1073) was mutated from CTTT-GAT to CTTGGGC. 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-NcoI-digested CRE-mutated luciferase vector. Clones were verified by restriction digests and sequencing.

Statistical Analysis—Data are presented as the means \pm 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.

RESULTS

Activation of PAR₂ Increases COX-2 Expression—We used the human lung carcinoma-derived A549 epithelial cell line as a model for PAR₂-induced COX-2 expression (14). We showed by immunoblotting that the selective PAR₂ activating peptide, SLIGRL-NH₂, but not the reverse-sequence inactive peptide, LRGILS-NH₂, induced a significant increase in COX-2 protein expression (Fig. 1A) 3 h after PAR₂ activation. The endogenous

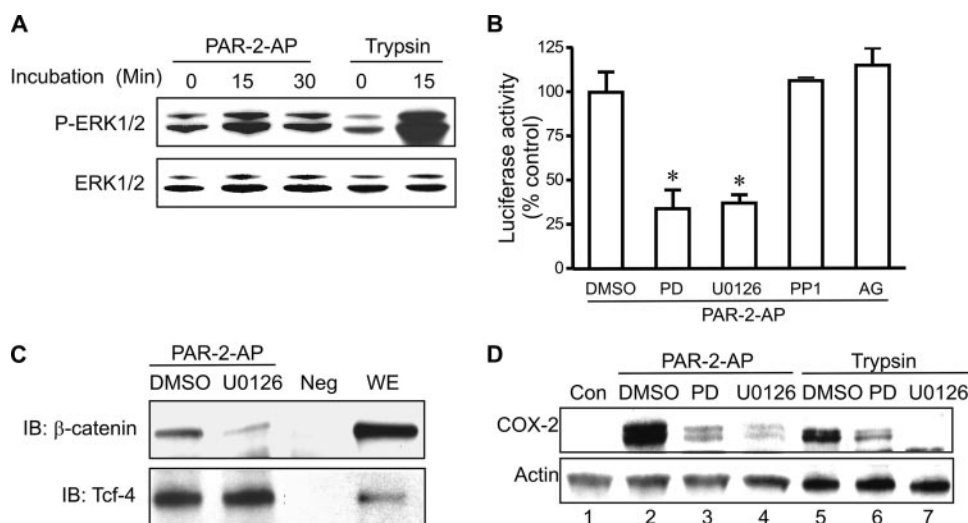


FIGURE 3. ERK1/2 is involved in PAR₂-induced β -catenin activation. *A*, A549 cells were treated with PAR₂-AP or trypsin for 0, 15, and 30 min. Cell lysates were prepared for Western blotting to detect phosphorylated (P) or total ERK1/2. *B*, cells were transfected with TOPFLASH or FOPFLASH combined with pRT-RL 1 day before the experiment. The cells were pretreated with MEK inhibitor PD98059 (PD; 50 μ M) or U0126 (50 μ M), Src inhibitor PP1 (1 μ M), or EGFR tyrosine kinase inhibitor AG1478 (AG; 10 μ M) 45 min before the stimulation with PAR₂-AP. Cells were then collected for luciferase assay. *, $p < 0.05$ compared with the vehicle control group (dimethyl sulfoxide (DMSO)) ($n \geq 3$). *C*, A549 cells were pretreated with Me₂SO or MEK inhibitor U0126 and then stimulated with PAR₂-AP for 3 h. After treatment, the nuclear fraction was collected, and immunoprecipitation was performed with an anti-Tcf-4 antibody. Western blotting was then conducted with anti- β -catenin and anti-Tcf-4 antibodies. Blotting with anti-Tcf-4 antibody was used as input control. Negative control (Neg) represents immunoblotting performed without antibody during the immunoprecipitation. A whole cell extract (WE) was loaded as a positive control. *D*, the whole cell lysate was collected 3 h after treatment with PAR₂-AP or trypsin, with or without the inhibitors of MEK, to measure the protein levels of COX-2 and actin. Con, control.

activators of PAR₂, trypsin and matriptase, also stimulated COX-2 protein expression (Fig. 1A). Furthermore, PAR₂ activation by PAR₂-AP, trypsin, and matriptase increased PGE₂ secretion at this time point (Fig. 1B), indicating that PAR₂ activation induced the expression of functional COX-2.

RT-PCR showed that the up-regulation of COX-2 enzyme triggered by PAR₂-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 PAR₂-AP-induced increase in COX-2 mRNA (Fig. 1D), indicating that PAR₂ induced COX-2 expression at the transcriptional level.

β -Catenin/Tcf-4 Pathway Mediates PAR₂-induced COX-2 Expression—Activation of PAR₂ 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 PAR₂-AP, trypsin, or matriptase, we observed a significant increase of coprecipitated β -catenin compared with control cells (Fig. 2B), indicating that PAR₂ activation increased the binding of β -catenin to Tcf-4 in the nucleus. To test whether the activation of β -catenin was required for PAR₂-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 PAR₂-induced COX-2 expression (Fig. 2D).

Because NF- κ B is also important for transcriptional regulation of COX-2, we tested whether NF- κ B was activated by PAR₂ signaling by using a κ B-driven luciferase reporter. Treatment

with tumor necrosis factor- α was used as a positive control for the activation of the NF- κ B pathway (Fig. 2E). PAR₂-AP at 25 and 50 μ M did not significantly change NF- κ B activity. However, when the concentration of PAR₂-AP was increased to 100 μ M, a concentration above that which activated β -catenin transcriptional activity, we did observe an elevation in κ B-luciferase activity (Fig. 2E).

PAR₂ Activation of β -Catenin Occurs through an ERK-dependent Pathway—The activation of PAR₂ by activating peptide (PAR₂-AP) or trypsin stimulated the ERK1/2 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 PAR₂-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 PAR₂-AP and trypsin was reduced

by the inhibition of ERK1/2 activation (Fig. 3D). These data indicated that ERK1/2 activation induced by PAR₂ led to β -catenin activation and COX-2 expression.

Inactivation of GSK-3 β by an ERK-dependent Pathway—GSK-3 β is part of the degradation complex that targets β -catenin to the proteasome. Phosphorylation of GSK-3 β at Ser-9 inhibits its activity, and β -catenin transcriptional activity subsequently increases. To test whether ERK1/2 induces β -catenin activation through inactivation of GSK-3 β by phosphorylation of Ser-9, we measured the inactivated (phosphorylated Ser-9) form of GSK-3 β by immunoblot analysis and found a significant accumulation of inactive GSK-3 β (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 PAR₂ activation (Fig. 4A). Significantly, the MEK inhibitor U0126 dramatically blocked the PAR₂-stimulated accumulation of both the inactivated phospho-GSK-3 β and active N-terminally dephosphorylated β -catenin (Fig. 4B). These results strongly suggested that PAR₂ caused an ERK1/2-mediated inhibition of GSK-3 β 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-NH₂ (Fig. 4C). However, PAR₂ activation resulted in a

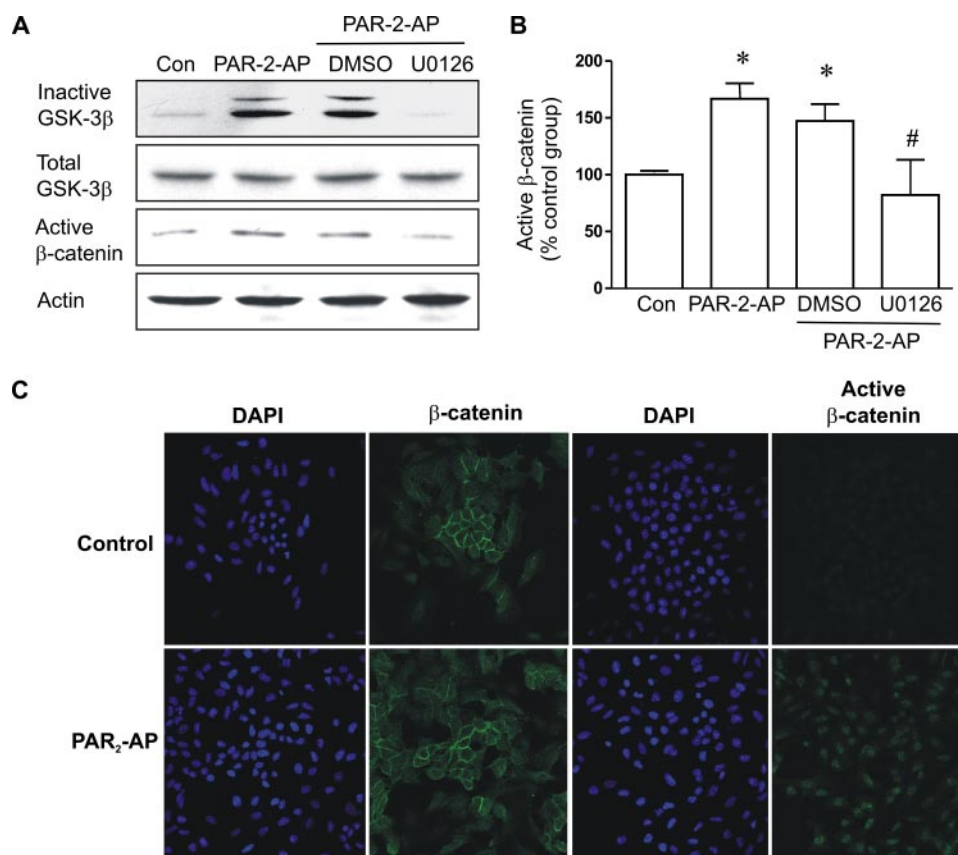


FIGURE 4. Activation of PAR₂ causes alterations in GSK-3 β and β -catenin activation states. *A*, the activated form of β -catenin and the inactivated form of GSK-3 β were detected by Western blotting. Inactive GSK-3 β and active β -catenin were increased following activation with PAR₂-AP. This effect was blocked by U0126. *B*, densitometry confirmed the PAR₂-induced increase in active β -catenin and the inhibition of this effect by U0126. *, $p < 0.05$ compared with the control group (Con); #, $p < 0.05$ compared with the PAR₂-AP-treated Me₂SO (DMSO) group ($n \geq 4$). *C*, immunocytochemistry showed no change in the cellular distribution of total β -catenin in control cells, but a substantial increase in nuclear immunoreactivity for active β -catenin following exposure to the PAR₂-AP. DAPI, 4',6-diamidino-2-phenylindole.

substantial increase in the nuclear localization of active β -catenin immunoreactivity (Fig. 4C).

EGFr Is Not Involved in PAR₂-induced β -Catenin Activation—EGFr has been shown to increase β -catenin nuclear translocation and transcriptional activity (28), and PAR₂ has been shown to transactivate EGFr through Src (29, 30). To test whether the activated EGFr might account for PAR₂-induced β -catenin activation, we used inhibitors of EGFr 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. 5A, lanes 1, 2, and 5). This effect was abolished by the inhibitors of MEK (PD98059 and U0126) (Fig. 5A). siRNA targeting CREB, which knocked down the protein level of CREB (Fig. 5B), significantly reduced PAR₂-induced COX-2 expression (Fig. 5C).

Crosstalk between β -Catenin and CREB—Interestingly, CREB siRNA not only blocked PAR₂-induced COX-2 expression but also abolished PAR₂-induced β -catenin activation

(Fig. 6A). 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 EGFr transactivation (14). Here we show that the activation of ERK1/2 is required for subsequent deactivation of GSK-3 β and activation of β -catenin transcriptional activity to induce COX-2 expression. It has been shown that ERK-

PAR₂ Stimulates COX-2 via β -Catenin

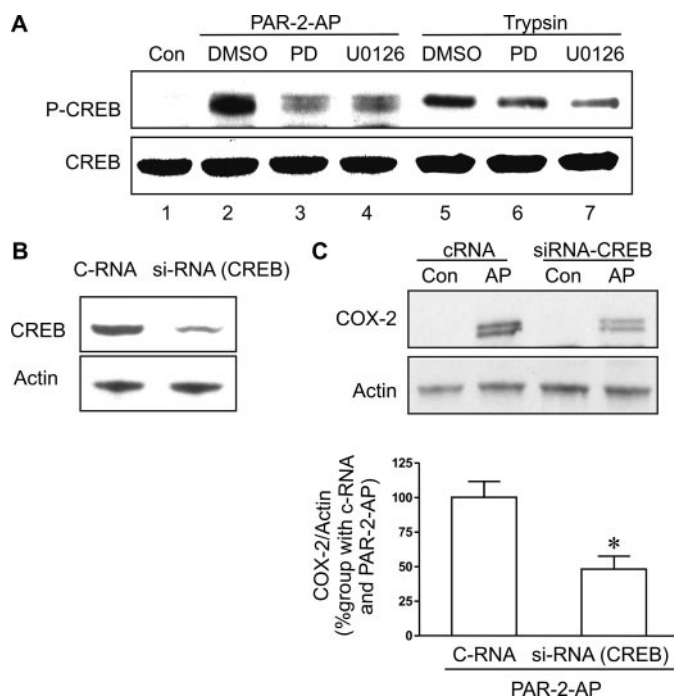


FIGURE 5. CREB is involved in PAR₂-induced COX-2 expression. *A*, after pretreatment with the vehicle (dimethyl sulfoxide (DMSO)) or the inhibitors of MEK (PD98059 (PD)) and U0126, A549 cells were challenged with PAR₂-AP or trypsin for 20 min. Cell lysates were then collected for measurement of phospho-CREB (Ser-133) (P-CREB) and total CREB by Western blotting. *Con*, control. *B*, cells were transfected with CREB siRNA or CONTROLTM RNA (C-RNA). Whole cell lysates were collected to measure CREB and actin by Western blotting. *C*, cells were transfected with CREB siRNA or CONTROLTM 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) ($n \geq 3$).

dependent phosphorylation of GSK-3 β at Ser-9 mediates β -catenin stabilization (36). Phosphorylation of GSK-3 β at Ser-9 is usually indicative of inactivation of this kinase (37) and is associated with increased β -catenin stability, although recent studies in mice in which GSK-3 β has been mutated suggest that this is not always the case (38). Interestingly, although based on our earlier study (14), Src-mediated EGFR transactivation seems to be required for COX-2 expression, it is not involved in the PAR₂-induced activation of β -catenin transcriptional activity, as demonstrated in the present study. Thus, there appear to be EGFR transactivation-dependent and -independent pathways involved in PAR₂-induced COX-2 expression. The EGFR-independent component involves β -catenin.

Several studies have implicated β -catenin and its nuclear binding proteins in COX-2 expression. COX-2 was down-regulated after the transfection of full-length APC into HT29 cells, which have truncated APC and thus constitutively high β -catenin (39). Further studies showed that β -catenin up-regulated COX-2 mRNA through transcriptional regulation by the Tcf-4 binding element (21) and mRNA stabilization (40) and that both the TBE and CRE are important in COX-2 expression (41, 42). Our findings have confirmed the critical role of β -catenin/Tcf-4 in the transcriptional regulation of COX-2 that follows PAR₂ activation.

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 pathway (43, 44). In the

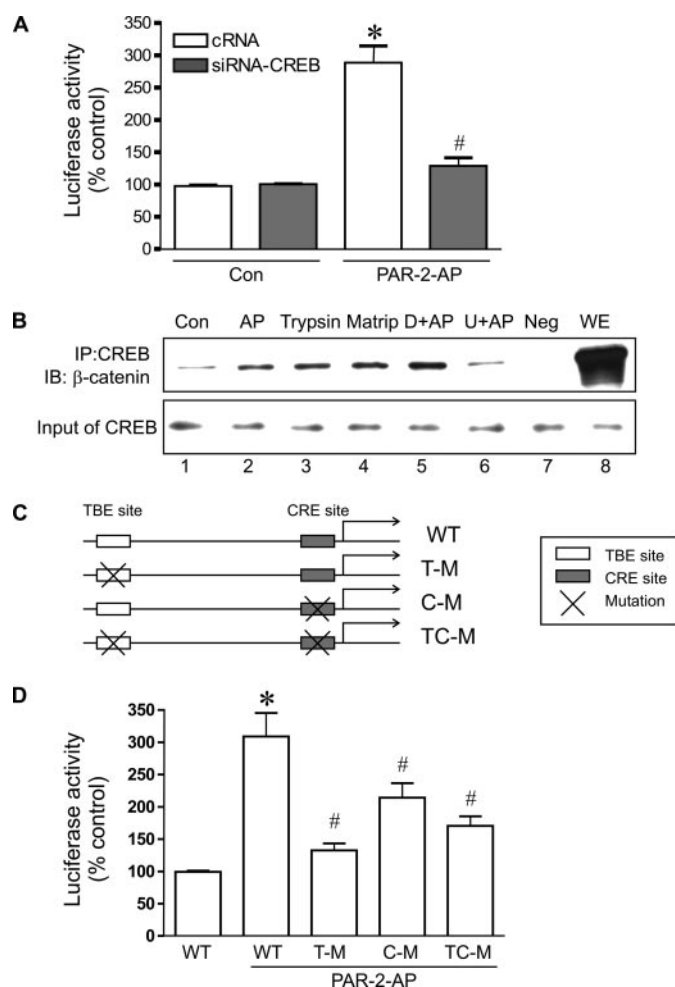


FIGURE 6. Interaction of β -catenin and CREB is required for PAR₂-induced COX-2 expression. *A*, A549 cells were transfected with siRNA against CREB or with CONTROLTM RNA (C-RNA) combined with TOPFLASH and pTK-RL. One day after transfection, the cells were treated with PAR₂-AP for 3 h, and the cell lysates 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 ($n \geq 3$). *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 CREB as an input control. The effect of activating peptide (AP) was blocked by the MEK inhibitor U0126 (U). *D*, Me₂SO control; *Con*, control; *IB*, immunoblot; *Matrip*, matriptase. *C*, the full-length wild-type (WT) COX-2 promoter reporter has TBE and CRE sites. PCR-based site-directed mutagenesis was used to build mutated COX-2 promoter reporters: TBE mutation (T-M), CRE mutation (C-M), or TBE and CRE mutations (TC-M). *D*, A549 cells were transfected with wild-type or mutated COX-2 promoter reporter and pTK-RL. One day after transfection, the cells were treated with or without PAR₂-AP for 3 h, and the cell lysates were collected for luciferase assay. *, $p < 0.05$ compared with the wild-type group; #, $p < 0.05$ compared with the PAR₂-AP-treated wild-type group ($n \geq 3$).

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.

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

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

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