Regulation of Cyclooxygenase-2 mRNA Stability by Taxanes. Evidence for Involvement of p38, MAPKAPK-2 and HuR.

Kotha Subbaramaiah*, Timothy P. Marmo*, Dan A. Dixonβ, and Andrew J. Dannenbergαβ

From the αDepartment of Medicine (Division of Gastroenterology and Hepatology), New York Presbyterian Hospital and Weill Medical College of Cornell University; *Strang Cancer Prevention Center, New York, New York 10021; βDepartments of Surgery and Cancer Biology, Vanderbilt University Medical Center, Nashville, Tennessee 37232

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Correspondence should be addressed to:
Kotha Subbaramaiah
New York Presbyterian Hospital-Cornell Campus
Department of Medicine
1300 York Avenue, Room F-203A
New York, N.Y. 10021
PH: (212)-746-4402
FAX: (212)-746-4885
Email: ksubba@med.cornell.edu
SUMMARY

Taxanes are widely used to treat malignancies and are known to modulate the transcription of several genes. We investigated the effects of taxanes (docetaxel, paclitaxel) on cyclooxygenase-2 (COX-2) transcription and mRNA stability in human mammary epithelial cells. As reported previously for paclitaxel, docetaxel stimulated COX-2 transcription by an AP-1-dependent mechanism. Treatment with taxanes also enhanced the stability of COX-2 mRNA. To define the mechanism by which taxanes stabilized COX-2 mRNA, transient transfections were carried out using luciferase expression constructs containing the COX-2 3’-untranslated region (3’-UTR). The stabilizing effects of taxanes were localized to the AU-rich region (ARE) of COX-2 3’-UTR. RNA-binding studies indicated that taxanes stimulated the binding of HuR to the AU-rich region of the COX-2 3’-UTR. Overexpression of antisense HuR suppressed taxane–mediated induction of COX-2 3’-UTR activity. We next investigated the signal transduction pathway responsible for taxane-mediated induction of COX-2. Taxanes enhanced protein kinase C (PKC) activity; overexpressing dominant negative PKC-α suppressed taxane-mediated stimulation of both COX-2 3’-UTR and 5’-promoter activities. Interestingly, ERK1/2, JNK and p38 MAPKs were important for taxane-mediated activation of COX-2 transcription but only p38 MAPK appeared to be responsible for the increase in COX-2 mRNA stability. MAPKAPK-2, a known target of p38 MAPK, contributed to increased COX-2 mRNA stability following taxane treatment. SB 202190, a selective p38 MAPK inhibitor, and dexamethasone suppressed taxane-mediated stimulation of the COX-2 3’-UTR and binding of HuR. Taken together, these data indicate that taxanes induce COX-2 by stimulating both transcription and mRNA
stability. To the best of our knowledge, this is the first evidence that taxanes can promote stabilization of mRNA in addition to modulating gene transcription.
INTRODUCTION

There are two isoforms of cyclooxygenase (COX) that catalyze the synthesis of prostaglandins (PGs) from arachidonic acid. COX-1 is constitutively expressed in most tissues and appears to mediate various physiological functions (1,2). By contrast, COX-2 is undetectable in most normal tissues but is rapidly induced by oncogenes, growth factors, cytokines, and tumor promoters (3-9).

COX-2 is an important pharmacological target for the treatment of arthritis and possibly cancer (10-13). Multiple lines of evidence suggest that COX-2 has a significant role in carcinogenesis. COX-2 is overexpressed in transformed cells (7,14) and in various malignancies (13,15-18). Overexpression of COX-2 in the mammary glands of transgenic mice resulted in enhanced tumorigenesis in multiparous mice (19). Moreover, mice engineered to be COX-2 deficient are protected against developing both intestinal (20,21) and skin tumors (22). Selective inhibitors of COX-2 reduce the formation, growth and metastasis of tumors in experimental animals and decrease the number of colorectal polyps in familial adenomatous polyposis patients (23-29). Several different mechanisms can potentially explain the link between COX-2 and malignancy. Increased synthesis of COX-2-derived PGs can stimulate cell proliferation (30), promote angiogenesis (31,32), increase invasiveness (33,34) and inhibit apoptosis (35,36). Importantly, treatment with chemotherapeutic agents such as taxanes induces COX-2 expression (37, 38) raising the possibility that cotreatment with a selective COX-2 inhibitor will augment the anti-tumor activity of chemotherapy (39,40).

Taxanes are widely used for the treatment of cancer. The anti-cancer properties of taxanes are due at least, in part, to interference with microtubule assembly, impairment of
mitosis, and changes in cytoskeleton (41). There is growing evidence, however, that
taxanes have multiple cellular effects. For example, taxanes stimulate mitogen-activated
protein kinases (MAPKs) and the expression of numerous genes including COX-2 (38,
42,43). However, there is limited knowledge about the effects of taxanes on the
molecular mechanisms regulating immediate-early gene expression.

The concentration of mRNA is determined by both rates of synthesis and
degradation (44). Although great emphasis has been placed on defining the mechanisms
that control COX-2 transcription (13), there is growing evidence that post-transcriptional
mechanisms are also important (45,46). It has become clear, for example, that an AU-
rich element (ARE) within the 3'-untranslated region (3'-UTR) of COX-2 mRNA can
affect both mRNA stability and protein translation (45-47). In this study, we investigated
whether taxanes (docetaxel, paclitaxel) induced COX-2 via effects on both message
stability and transcription. Evidence is presented that taxanes stimulated COX-2
transcription by enhancing the binding of AP-1 to the cyclic AMP response element
(CRE) of the COX-2 promoter. Additionally, taxanes stabilized COX-2 message by
enhancing the binding of the mRNA stabilization factor HuR to the ARE of the COX-2
3'-UTR. Remarkably, different MAPK signaling pathways were important for mediating
the effects of taxanes on COX-2 transcription vs. message stability. In addition to
modulating transcription, these findings provide the first evidence that taxanes can alter
post-transcriptional mechanisms.
EXPERIMENTAL PROCEDURES

Materials- Minimum Eagle's medium, PKC assay kits and LipofectAMINE were from Life Technologies, Inc. (Carlsbad, CA). Keratinocyte basal medium (KBM) was from Clonetics Corp. (San Diego, CA). Paclitaxel, sodium arachidonate, hydrocortisone, dexamethasone, actinomycin D, o-nitrophenyl-β-D-galactopyranoside, nocodazole and cytochalasin D were from Sigma (St. Louis, MO). SP 600125 was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). PD 98059 (2'-amino-3'-methoxyflavone) and SB 202190 (4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole) were from Calbiochem (La Jolla, CA). Enzyme immunoassay reagents for PGE2 assays were from Cayman Co. (Ann Arbor, MI). [32P]ATP, [32P]CTP, and [32P]UTP were from NEN Life Science Products (Boston, MA). Random-priming kits were from Roche Molecular Biochemicals (Indianapolis, IN). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). Reagents for the luciferase assay were from Analytical Luminescence (San Diego, CA). The 18 S rRNA cDNA was from Ambion, Inc. (Austin, TX). Antisera for human COX-2, NF-κB (p65), NF-IL6, β-actin, PEA3 and HuR were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to ERK1/2, p38, c-Jun, phospho-ERK1, phospho-p38, phospho-c-Jun and phospho-MAPKAPK-2 (MK-2) were from Cell Signaling Technology (Beverly, MA). Western blotting detection reagents (ECL) were from Amersham Pharmacia Biotech (Piscataway, NJ). In vitro transcription kits were from Promega Corp. (Madison, WI). Docetaxel was provided by Aventis Pharmaceuticals, Inc. (Bridgewater, NJ).

Tissue Culture- The 184B5/HER cell line has been described previously (48). Cells were maintained in Minimum Eagle's medium-KBM mixed in a ratio of 1:1 (basal...
medium) containing epidermal growth factor (10 ng/ml), hydrocortisone (0.5 µg/ml), transferrin (10 µg/ml), gentamicin (5 µg/ml), and insulin (10 µg/ml) (growth medium). Cells were grown to 60% confluence, trypsinized with 0.05% trypsin-2 mM EDTA, and plated for experimental use. In all experiments, cells were grown in basal medium for 24 h before treatment. Treatment with vehicle (0.1% Me₂SO) or taxane was always carried out in basal medium.

**PGE₂ Production** - 5 X 10⁴ cells/well were plated in 6-well dishes and grown to 60% confluence in growth medium. Levels of PGE₂ released by the cells were measured by enzyme immunoassay. Production of PGE₂ was normalized to protein concentrations.

**Western Blotting** - Cell lysates were prepared by treating cells with lysis buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml trypsin inhibitor, and 10 µg/ml leupeptin). Lysates were sonicated for 20 s on ice and centrifuged at 10,000 X g for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry et al. (49). SDS-polyacrylamide gel electrophoresis was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (50). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (51). The nitrocellulose membrane was then incubated with primary antisera. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with the ECL Western blot detection system according to the manufacturer's instructions.

**Northern Blotting** - Total cellular RNA was isolated from cell monolayers using an RNA isolation kit from Qiagen Inc. (Valencia, CA). 10 µg of total cellular RNA per
lane were electrophoresed in a formaldehyde-containing 1.2% agarose gel and transferred to nylon-supported membranes. After baking, membranes were prehybridized overnight in a solution containing 50% formamide, 5X sodium chloride/sodium phosphate/EDTA buffer (SSPE), 5XDenhardt's solution, 0.1% SDS, and 100 µg/ml single-stranded salmon sperm DNA and then hybridized for 12 h at 42°C with radiolabeled cDNA probes. After hybridization, membranes were washed twice for 20 min at room temperature in 2X SSPE, 0.1% SDS, twice for 20 min in the same solution at 55 °C, and twice for 20 min in 0.1X SSPE, 0.1% SDS at 55°C. Washed membranes were then subjected to autoradiography. COX-2 (4.3 Kb), luciferase (1.6 Kb) and 18S rRNA (1.6 Kb) probes were labeled with [32P]CTP by random priming.

**Analysis of Protein-RNA Interactions**—The COX-2 ARE sequence cloned into pBlueScript (KS+) (45) was used in *in vitro* transcription reactions incorporating [32P]UTP (50 µCi) in sense RNAs for COX-2. Unlabeled competitor RNAs were made using the Ribomax kit (Promega). Cytoplasmic cell lysates were prepared as previously described (45). Cells were grown in T150 tissue culture dishes, and washed twice with phosphate-buffered saline before 4 ml of lysis buffer (25 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40) was added. Thawed cells were scraped from the plate, vortexed briefly, and centrifuged at 14,000 X g for 10 min. The supernatant was assayed for protein concentration using a Lowry protein assay with bovine serum albumin as standard and used immediately or snap frozen at -70°C. For native gel mobility shift assay, 5 µg of cytoplasmic lysate was incubated with radiolabeled RNA in binding buffer (20 mM HEPES (pH 7.5), 3 mM MgCl₂, 40 mM KCl, 1 mM dithiothreitol, 5% glycerol) in a total volume of 20 µl. The mixture was then incubated for 15 min at room temperature prior to
the addition of heparin (5 mg/ml). Following incubation for an additional 20 min, samples were electrophoresed in 4% polyacrylamide gels (60:1 acrylamide/bisacrylamide) in 0.5X TBE (Tris borate-EDTA) buffer containing 5% glycerol. The gel was dried and exposed overnight with Kodak Bio-Max MS film and an intensifying screen.

**Nuclear Run-off Assay**-- 2.5 X 10^5 cells were plated in four T150 dishes for each condition. Cells were grown in growth medium until approximately 60% confluent. Nuclei were isolated and stored in liquid nitrogen. For the transcription assay, nuclei (1.0 X 10^7) were thawed and incubated in reaction buffer (10 mM Tris (pH 8), 5 mM MgCl2, and 0.3 M KCl) containing 100 µCi of [32P]UTP and 1 mM unlabeled nucleotides. After 30 min, labeled nascent RNA transcripts were isolated. The human COX-2 and 18 S rRNA cDNAs were immobilized onto nitrocellulose and prehybridized overnight in hybridization buffer. Hybridization was carried out at 42°C for 24 h using equal cpm/ml labeled nascent RNA transcripts for each treatment group. The membranes were washed twice with 2X SSC buffer for 1 h at 55°C and then treated with 10 mg/ml RNase A in 2X SSC at 37°C for 30 min, dried, and autoradiographed.

**Plasmids**-The COX-2 5’UTR promoter constructs (-1432/+59, -327/+59, -220/+59, -124/+59, -52/+59, KBM, ILM, CRM) were a generous gift of Drs. Tadashi Tanabe and Hiroyasu Inoue (National Cardiovascular Center Research Institute, Osaka, Japan). The COX-2 3’-UTR constructs have been described previously (45,52). Dr. Stephen M. Prescott (University of Utah, Salt Lake City, UT) generously provided the human COX-2 cDNA. The ERK1 DN expression vector was obtained from Dr. Melanie Cobb (Southwestern Medical Center, Dallas, TX). Expression vectors for JNK, p38 and
MK-2 were generously provided by Dr. Roger Davis (University of Massachusetts, Worcester, MA). HuR expression vectors were obtained from Dr. Joan Steitz (Yale University, New Haven, CT). PKC-α wild-type and dominant negative constructs were obtained from Dr. I.B. Weinstein (College of Physicians and Surgeons of Columbia University, New York, NY). pSVβgal was obtained from Promega Corp.

**Oligonucleotides**—Oligonucleotides containing different COX-2 promoter sites were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX). CRE, 5′-AACAGTCATTTTCGCAATACATGGCCTTG-3′ (sense), 5′-CAAGCCCATGTGACGAAATGACTTTT-3′ (antisense); mutant CRE, 5′-AACAGTCATTTTCGCAATACATGGCCTTG-3′ (sense), 5′-CAAGCCCATGTGACGAAATGACTTTT-3′ (antisense); NF-κB, 5′-AGGAGAGTGCTTGGACTACCCCTCTCTCTG-3′ (sense), 5′-AGGAGAGTGCTTGGACTACCCCTCTCT-3′ (antisense); NF-IL6, 5′-ACCCCGGGCTTACGCAATT-3′ (sense), 5′-AATTGCGTAAGCCCGGTGGG-3′ (antisense); PEA3, 5′-AGGCGGAAAGAA-3′ (sense) 5′-TTCTTTCCGCTTTTTCCGTACC-3′ (antisense).

**Transient Transfection Assays**—Cells were seeded at a density of 5 X 10⁴ cells/well in 6-well dishes and grown to 50-60% confluence. For each well, 2 µg of plasmid DNA were introduced into cells using 8 µg of LipofectAMINE as per manufacturer's instructions. After 7 h of incubation, the medium was replaced with basal medium. Reporter activities were measured at the indicated times.

**Protein Kinase C Assay**—The activity of PKC was measured according to directions from Life Technologies, Inc. Briefly, cells were plated in 10-cm dishes at 10⁶ cells/dish and grown to 60% confluence. Cells were then treated with fresh basal medium containing vehicle (0.2% Me₂SO) or taxane (0-10 µM), for 6h. Total PKC activity was
measured in cell lysates as per the manufacturer’s instructions. Briefly, PKC activity was measured by incubating partially purified PKC with $^{32}\text{P}}\text{ATP (3000-6000 Ci/mmol)}$ and the substrate myelin basic protein for 20 min at room temperature. The activity of PKC is expressed as cpm incorporated/µg protein.

Statistics-Comparisons between groups were made by the Student's $t$-test. A difference between groups of $p < 0.05$ was considered significant.
RESULTS

**Docetaxel stimulates the transcription of COX-2.** Treatment of the mammary epithelial cell line 184B5/HER with docetaxel caused dose dependent induction of PGE2 biosynthesis (Fig. 1A). 10 μM docetaxel led to 6.3-fold induction of PGE2. Western blotting was carried out to determine whether the differences in PGE2 production correlated with amounts of COX-2. Consistent with the observed increase in PG biosynthesis, docetaxel was a potent inducer of COX-2 protein (Fig. 1B). COX-1 was not detectable by immunoblotting in this cell line (data not shown). To further elucidate the mechanism responsible for the changes in amounts of COX-2 protein, we examined steady-state levels of COX-2 mRNA by Northern blotting. As shown in Fig. 1C, higher levels of COX-2 mRNA were also detected following treatment with docetaxel. To investigate whether docetaxel regulated the transcription of COX-2, nuclear run-offs were performed. Higher rates of synthesis of nascent COX-2 mRNA were observed after treatment with docetaxel (Fig. 1D).

We next were interested in identifying the region of the COX-2 promoter that was important for mediating the inductive effects of docetaxel. Transient transfections were performed with a series of human COX-2 5′-promoter-deletion constructs (Fig. 2A). In agreement with the results shown in Fig. 1D, treatment of cells with docetaxel led to a doubling of COX-2 promoter activity when a -1432/+59 COX-2 promoter construct was utilized (Fig. 2B). A stepwise decrease in basal COX-2 promoter activity was observed when shorter constructs were used. However, the magnitude of induction by docetaxel remained essentially constant with all promoter deletion constructs except the -52/+59 construct (Fig. 2B). The -52/+59 COX-2 promoter construct was not stimulated by docetaxel. This result implies that one or more promoter elements lying between −53 and
–123 are necessary for docetaxel-mediated induction of COX-2. A CRE is present between nucleotides -59 and -53 raising the possibility that this element could be involved in mediating the inductive effects of docetaxel. To test this notion, transient transfections were performed utilizing COX-2 promoter constructs in which specific known enhancer elements including the CRE were mutagenized. As shown in Fig. 2C, mutagenizing the CRE site abrogated docetaxel-mediated stimulation of COX-2 promoter activity. By contrast, mutagenizing the NFκB or NF-IL6 sites had no effect on COX-2 promoter function.

Electrophoretic mobility shift assays were performed to identify the transcription factor that contributed to docetaxel-mediated induction of COX-2. Increased binding of nuclear proteins to the CRE site of the COX-2 promoter was detected (Figs. 3A and 3B). In contrast, docetaxel did not increase binding to either the NF-IL6 or NF-κB sites of the COX-2 promoter (Fig. 3A). The increase in binding to the COX-2 CRE was competed by incubating nuclear extract from docetaxel-treated cells with an excess of CRE or AP-1 cold probes (data not shown) but not NF-κB, NF-IL6, PEA3 or mutant CRE cold probes (Fig. 3C). Supershift analysis identified c-Jun, c-Fos and ATF-2 in the binding complex (Figs. 3D and 3E). A supershift was not observed with antibodies to NF-κB p65, NF-IL6 or PEA3 (Fig. 3D). These findings are consistent with prior evidence that microtubule interfering agents including paclitaxel stimulate AP-1-mediated activation of COX-2 transcription via the CRE (38).

**Taxanes stabilize COX-2 mRNA.** We also investigated whether docetaxel or paclitaxel stabilized COX-2 mRNA. Cells were treated with vehicle or taxane for 3h to induce COX-2 transcription, and then transcription was stopped with the addition of actinomycin D. RNA was isolated at different time points after treatment with actinomycin D and subjected to
Northern blot analysis (Figs. 4A & 4B). As shown in Fig. 4C, treatment with either
docetaxel or paclitaxel caused a significant increase in the half-life of COX-2 mRNA.

Recently, an ARE was identified in the proximal end of the 3'-UTR of COX-2
mRNA (45). Because the ARE is important for modulating COX-2 mRNA stability,
experiments were carried out to determine whether it mediated the stabilizing effects of
docetaxel. Transient transfections were carried out with expression constructs containing
luciferase ligated to the full-length COX-2 3'-UTR, the ARE region, or the ARE deleted
from the full-length 3'-UTR (Fig. 5A). Treatment with docetaxel caused about a doubling
of luciferase activity when either the full length COX-2 3'-UTR or the ARE containing
construct were used (Fig. 5B). In contrast, docetaxel did not stimulate luciferase activity
when the ARE was deleted from the full length COX-2 3'-UTR. Corresponding changes in
amounts of luciferase mRNA were detected (Fig. 5C). Similar results were obtained with
paclitaxel (data not shown). Taken together, these results suggest that the ARE of the COX-
2 3'-UTR is responsible for mediating the increase in COX-2 message stability following
treatment with taxane.

Post-transcriptional regulation mediated by AREs is facilitated by trans-acting
factors that bind to the ARE. Hence, we investigated whether treatment with taxanes altered
binding of cytosolic RNA binding proteins to the COX-2 ARE. To evaluate this, we
incubated cytoplasmic lysates with in vitro transcribed 32P-labeled RNA containing the
COX-2 ARE. As shown in Fig. 5D, treatment with docetaxel caused a marked increase in
binding of cytoplasmic factors to the COX-2 ARE. Importantly, the docetaxel-mediated
increase in binding to the COX-2 ARE was competed by an excess of cold ARE but not by
mutant ARE or other regions of the 3'-UTR (Fig. 5E). In addition, docetaxel treatment did
not augment binding to either mutant ARE or to other regions of the 3'-UTR (Fig. 5E) thereby confirming the specificity of the protein binding to the COX-2 ARE. We next attempted to identify the factors responsible for increased COX-2 ARE binding following treatment of cells with docetaxel. To accomplish this, supershift assays were performed and the ARE-binding protein HuR was identified as the major protein in the binding complex that was induced by treatment with docetaxel (Fig. 6A). In contrast, normal IgG did not cause a supershift (Fig. 6B). To confirm the functional importance of HuR for mediating the stabilizing effects of docetaxel, additional experiments were performed. Overexpressing antisense HuR resulted in reduced levels of HuR (Fig. 6C) and inhibited docetaxel-mediated stimulation of the COX-2 3'-UTR (Fig. 6D). Corresponding changes in amounts of luciferase mRNA were detected (Fig. 6E). Comparable results were obtained with paclitaxel (data not shown).

**Defining the signaling mechanism by which docetaxel induces COX-2.** Activation of PKC is known to induce COX-2 (13). Hence, it was of interest to determine whether treatment with docetaxel or paclitaxel enhanced PKC activity. Treatment with either taxane induced PKC activity. As shown in Fig. 7A, docetaxel caused a dose-dependent increase in PKC activity. Transient transfections were performed to determine whether taxane-mediated stimulation of PKC activity contributed to the induction of COX-2. Overexpressing a dominant negative form of PKC-α abrogated the activation of both the COX-2 5'-UTR and the COX-2 3'-UTR by docetaxel (Fig. 7B). Changes in amounts of luciferase mRNA corresponded to the effects on the COX-2 3'-UTR (Fig. 7C). Similar results were obtained when paclitaxel was used (data not shown). Previous studies have demonstrated that MAPKs can be important for regulating both COX-2 transcription and mRNA stability.
Interestingly, docetaxel stimulated the activities of ERK1/2, JNK and p38 MAPKs (Fig. 8A-C). Subsequently, experiments were done to determine whether increased MAPK activity was linked to elevated levels of COX-2 in docetaxel-treated cells. In the first experiment, we utilized PD 98059, a specific inhibitor of MAPK kinase, which prevents activation of ERK1 and ERK2. Treatment with PD 98059 caused a decrease in docetaxel-mediated induction of COX-2 (Fig. 8D). Similarly, SB 202190, a selective inhibitor of p38 MAPK, suppressed the inductive effects of docetaxel (Fig. 8D). An inhibitor of JNK activity also suppressed docetaxel-mediated induction of COX-2 (Fig. 8E). For each inhibitor, changes in amounts of COX-2 correlated with changes in the activities of MAPKs (Fig. 8F-H). These results clearly indicate that multiple MAPKs are important for docetaxel-mediated induction of COX-2. Because docetaxel induces COX-2 by stimulating transcription and stabilizing message, additional experiments were carried out to define the role of specific MAPKs in regulating these different effects. As shown in Fig. 9, dominant negative forms of ERK1 and JNK were effective in suppressing docetaxel-mediated activation of the COX-2 5’-UTR but not the COX-2 3’-UTR. By contrast, the dominant negative form of p38 MAPK abrogated the inductive effects of docetaxel on the COX-2 3’-UTR while having a much more modest inhibitory effect on the activation of the COX-2 5’-UTR. Changes in luciferase mRNA corresponded to the observed changes in COX-2 3’-UTR activity (Fig. 9C). The above results strongly suggest that p38 MAPK is very important for stabilizing COX-2 message following treatment with docetaxel. In comparable experiments, p38 MAPK also appeared to mediate enhanced COX-2 message stability following treatment with paclitaxel (data not shown).
Additional experiments were carried out to further investigate the importance of p38 MAPK signaling in regulating COX-2 message stability. p38 activates MK-2 (53). As shown in Fig. 10, docetaxel induced MK-2 activity (Fig. 10A) and a dominant negative form of MK-2 blocked docetaxel-mediated stimulation of COX-2 3′UTR activity (Fig. 10B). Corresponding changes in amounts of luciferase mRNA were observed (Fig. 10C). Similar effects were observed when paclitaxel was used (data not shown). As shown in Figs. 11A and 11B, the inductive effects of docetaxel on the COX-2 3′-UTR and levels of luciferase mRNA were suppressed by treatment with a selective inhibitor of p38 MAPK. Consistent with this finding, docetaxel-mediated induction of HuR binding to the COX-2 3′-UTR was also suppressed by the inhibitor of p38 MAPK (Fig. 11C). This effect was specific because PD 98059, a compound that blocks the activation of ERK1/2, failed to alter the increase in HuR binding mediated by docetaxel (Fig. 11C). Dexamethasone has been reported to destabilize COX-2 mRNA (45). Hence, it was also of interest to evaluate whether dexamethasone could alter the mRNA stabilizing effects of docetaxel. Dexamethasone blocked docetaxel-mediated activation of the COX-2 3′-UTR (Fig. 11A), caused a corresponding change in amounts of luciferase mRNA (Fig. 11B), and inhibited the binding of HuR to the COX-2 ARE (Fig. 11C).

Taxane-mediated stabilization of COX-2 mRNA could be mediated by effects on the cytoskeleton. To further evaluate this concept, it was important to investigate the effects of nocodazole, a prototypic microtubule interfering agent. As shown in Fig. 12, nocodazole stimulated binding to the ARE (Fig. 12A) and induced COX-2 3′-UTR activity via the ARE (Fig. 12B). A corresponding change in levels of luciferase mRNA was observed (Fig. 12C).
Similar effects were observed when cytochalasin D, an inhibitor of actin polymerization was used (data not shown).
DISCUSSION

COX-2 is regulated by both transcriptional and post-transcriptional mechanisms. Here we have shown that taxanes induce COX-2 expression by both stimulating transcription and stabilizing mRNA. Docetaxel stimulated COX-2 transcription via the PKC→MAPK→AP-1 pathway. Previously, we showed that other microtubule interfering agents including paclitaxel activated COX-2 transcription by a similar mechanism (38).

To the best of our knowledge, the observation that both docetaxel and paclitaxel stabilized COX-2 mRNA represents the first evidence that taxanes can alter mRNA stability. Several recent studies have shown that an ARE within the 3′-UTR of COX-2 mRNA is important for regulating message stability (45-47, 54). Consistent with these prior studies, we found that the ARE within the proximal 3′-UTR of COX-2 mRNA was responsible for the increase in COX-2 message stability mediated by taxanes. HuR, a member of the ELAV (embryonic lethal abnormal vision) family of RNA-binding proteins (55,56), has been identified as a trans-acting factor that stabilizes messages containing AREs. In fact, HuR was recently shown to bind to the COX-2 ARE and decrease message turnover (46). The current results suggest that HuR is responsible for the increase in COX-2 message stability following treatment with taxanes. More specifically, treatment with taxanes increased the binding of HuR to the ARE of COX-2 3′-UTR. The functional significance of HuR was established because taxane-mediated induction of COX-2 3′-UTR activity was suppressed by overexpressing antisense HuR. HuR can stabilize mRNAs encoding a variety of other proteins implicated in inflammation and carcinogenesis including c-Fos, p21, vascular endothelial growth
factor, and cyclins A and B (57-59). The results of the current experiments suggest that it will be worthwhile to determine whether taxanes regulate the expression of these or other ARE containing messages.

It was important to elucidate the signaling mechanism(s) that mediated the induction of COX-2 by docetaxel and paclitaxel (Fig. 13). Treatment with taxanes caused a dose-dependent increase in PKC activity. Importantly, overexpression of a dominant negative form of PKC-α blocked taxane-mediated stimulation of COX-2 5’-UTR and 3’-UTR activity. This implies that PKC signaling is important for both the transcriptional and post-transcriptional regulation of COX-2. Notably, another recent study also demonstrated a role for PKC in regulating COX-2 transcription and message stability (60). Taxane treatment also activated ERK1/2, JNK and p38 MAPK; this can be explained at least, in part, by enhanced PKC signaling. There is considerable evidence that MAPKs are important for regulating both COX-2 transcription and mRNA stability. Less is known, however, about potential differences in the role of individual MAPKs in regulating these different control mechanisms. It was of interest, therefore, to determine whether the same MAPKs were required for taxane-mediated induction of COX-2 transcription vs. increased message stability. Inhibitors of MAPK kinase, JNK and p38 MAPK blocked the induction of COX-2 by docetaxel. Moreover, overexpression of dominant negatives for ERK1, JNK or p38 MAPK suppressed the induction of COX-2 5’-promoter activity by taxane. These results suggest the involvement of multiple MAPKs in regulating taxane-mediated induction of COX-2 transcription. In contrast, p38 MAPK was principally responsible for the observed increase in COX-2 mRNA stability (61). This conclusion is supported by several findings. First overexpression of a dominant
negative form of p38 blocked taxane-induced stimulation of COX-2 3’-UTR activity. In contrast, overexpressing dominant negative forms of ERK1 and JNK failed to suppress taxane-mediated activation of the COX-2 3’UTR although they effectively inhibited the induction of COX-2 5’-promoter activity. Two other findings support a pivotal role for p38 MAPK in regulating COX-2 message stability. SB 202190, a selective inhibitor of p38 MAPK, blocked both taxane-mediated stimulation of COX-2 3’-UTR activity and the increase in HuR binding to the COX-2 3’-UTR. In addition to taxanes, various other agents (e.g., nocodazole, cytochalasin D) affect the cytoskeleton and stimulate p38 MAPK activity (38). It is noteworthy, therefore, that both nocodazole and cytochalasin D stimulated COX-2 3’-UTR activity. This finding supports the concept that the cytoskeleton is important for propagating signals that regulate mRNA stability in addition to gene transcription.

MK-2 mediates the effects of p38 MAPK on mRNA stability (62). Taxanes stimulated MK-2 activity; a dominant negative form of MK-2 blocked taxane–mediated induction of COX-2 3’UTR activity. Hence, p38 stabilizes COX-2 mRNA via MK-2 activation (Fig. 13). The precise mechanism by which MK-2 modulates mRNA stability is not well understood. However, there are at least two possible mechanisms by which MK-2 can increase COX-2 mRNA stability. One potential downstream effector of MK-2 is HSP27, which is a substrate of MK-2 (54). Studies have shown that a phospho-mimetic mutant of HSP27 stabilizes β-globin-COX-2 3’-UTR mRNA in transfected cells (54). A second possibility is that another substrate of MK-2, hnRNP A0, is responsible for taxane–mediated stabilization of COX-2 mRNA. This possibility is suggested by the recent finding that LPS-mediated stabilization of COX-2 mRNA stability is dependent on MK-2
activation of hnRNP A0 (63). Further work will be required to define the mechanism(s) by which stimulation of p38 MAPK leads to enhanced binding of HuR to the COX-2 3’-UTR resulting in increased message stability.

Previously, dexamethasone was reported to decrease COX-2 message stability (64). Dexamethasone inhibits p38 MAPK activity by inducing MAPK phosphatase-1 (MKP-1) (65). Taxane-mediated activation of the COX-2 3’-UTR and HuR binding was inhibited by dexamethasone. Hence, it will be of considerable interest to investigate whether this is a consequence of enhanced MKP-1 activity. Steroids are given to prophylax against the side effects of taxane treatment. In all likelihood, the ability of steroids to prevent HuR-induced stabilization of ARE containing mRNAs including COX-2 contributes to the anti-inflammatory properties of these agents.

Previously, we postulated that taxane-mediated induction of COX-2 in tumors might decrease the efficacy of this form of chemotherapy (38). Accordingly, we suggested that coadministration of a selective COX-2 inhibitor might increase the efficacy of taxane-based chemotherapy. In support of this notion, a selective COX-2 inhibitor was recently found to enhance the efficacy of docetaxel in the treatment of experimental lung cancer (39). Although taxanes are effective anti-tumor agents, tumor resistance remains a common problem. In this study, we show for the first time that taxanes enhance COX-2 message stability by augmenting HuR binding. Undoubtedly, other mRNAs that are regulated by HuR will also be stabilized. This mechanism could contribute to tumor resistance. As newer agents are developed that can impact on this mechanism, e.g., selective p38 MAPK inhibitors, it will be important to consider evaluating them in combination with taxane-based chemotherapy regimens.
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FOOTNOTES

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The abbreviations used are COX, cyclooxygenase; PG, prostaglandin; MAPK, mitogen-activated protein kinase; ARE, AU-rich element; UTR, untranslated region; CRE, cyclic AMP response element; MK-2, MAPK-activated protein kinase-2; MKP-1, MAPK phosphatase-1; PKC, protein kinase C; DN, dominant negative
**LEGENDS TO FIGURES**

**Figure 1. Docetaxel stimulates the transcription of COX-2.** A, 184B5/HER cells were treated with 0-10 µM docetaxel for 6 h. The medium was then replaced with basal medium containing 10 µM sodium arachidonate. 30 min later, the medium was collected to determine amounts of PGE2. Production of PGE2 was determined by enzyme immunoassay. Columns, means; bars, S.D.; n=6. B, cellular lysate protein (25 µg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibody specific for COX-2. Cell lysates were prepared from cells treated with vehicle (lane 2) or docetaxel (1, 2.5, 5 and 10 µM; lanes 3-6) for 6 h. Lane 1 represents an ovine COX-2 standard. C, total cellular RNA was isolated from cells treated with vehicle (lane 1) or docetaxel (1, 2.5, 5, 10 µM; lanes 2-5) for 3 h. 10 µg of RNA was added to each lane. The blot was hybridized with probes that recognized COX-2 mRNA and 18S rRNA. D, nuclei were isolated from cells treated with vehicle (lane 1) or 10 µM docetaxel (lane 2) for 3 h. The COX-2 and 18S rRNA cDNAs were immobilized onto nitrocellulose membranes and hybridized with labeled nascent RNA transcripts.

**Figure 2. Localization of region of COX-2 promoter that mediates the effects of taxotere.** A, shown is a schematic of the human COX-2 promoter. B, 184B5/HER cells were transfected with 1.8 µg of a series of human COX-2 promoter deletion constructs ligated to luciferase (-1432/+59, -327/+59, -220/+59, -124/+59, -52/+59) and 0.2 µg pSVβgal. C, cells were transfected with 1.8 µg of a series of human COX-2 promoter-luciferase constructs (-327/+59; KBM; ILM; CRM) and 0.2 µg pSVβgal. KBM represents...
the -327/+59 COX-2 promoter construct in which the NFκB site was mutagenized; ILM represents the -327/+59 COX-2 promoter construct in which the NF-IL6 site was mutagenized; CRM refers to the -327/+59 COX-2 promoter construct in which the CRE was mutagenized. After transfection, cells were treated with vehicle or 10 μM docetaxel. Reporter activities were measured in cellular extract 7 h later. Luciferase activity represents data that have been normalized with β-galactosidase. Columns, means; bars, SD; n=6.

Figure 3. Increased binding of c-Jun, c-Fos and ATF-2 to the CRE of the COX-2 promoter is detected in docetaxel-treated cells. In A-E, 5 μg of nuclear protein from 184B5/HER cells was incubated with a 32P-labeled oligonucleotide. A, nuclear protein was isolated from cells treated for 30 min with vehicle (lanes 1, 3, 5) or 10 μM docetaxel (lanes 2, 4, 6). Nuclear protein was incubated with a 32P-labeled oligonucleotide containing the NF-κB (lanes 1, 2), NF-IL6 (lanes 3, 4) or CRE (lanes 5, 6) sites of COX-2. B, cells were treated with vehicle (lane 1) or docetaxel (5 and 10 μM; lanes 2 and 3) for 30 min. Nuclear extracts were incubated with a 32P-labeled COX-2 CRE oligonucleotide. C, cells were treated with vehicle (lane 1) or 10 μM docetaxel (lanes 2-7). Nuclear extracts were incubated with a 32P-labeled COX-2 CRE oligonucleotide (lanes 1, 2). In lanes 3-7, nuclear extract from docetaxel-treated cells was incubated with a 32P-labeled COX-2 CRE oligonucleotide and a 100-fold excess of unlabeled oligonucleotide containing NF-κB (lane 3), NF-IL6 (lane 4), PEA3 (lane 5), mutant CRE (lane 6) or CRE (lane 7) sites of COX-2. D, cells were treated with vehicle (lane 1) or 10 μM docetaxel (lanes 2-7) for 30 min. Nuclear extracts were incubated with a 32P-labeled oligonucleotide containing the CRE of COX-2. In lanes 3-7, supershift assay was performed by incubating nuclear extract from
docetaxel-treated cells with antibodies to NF-κB p65 (lane 3), NF-IL6 (lane 4), PEA3 (lane 5) or c-Jun (lane 7). Lane 6 represents IgG. E, cells were treated for 30 min with 10 μM docetaxel. Nuclear extracts were incubated with a ^32P-labeled COX-2 CRE oligonucleotide. Lane 1 represents nuclear extract from docetaxel-treated cells; lanes 2-4 represent nuclear extracts from docetaxel-treated cells incubated with antibodies to c-Jun (lane 2), c-Fos (lane 3) and ATF-2 (lane 4), respectively. In A-E, the protein-DNA complexes that formed were separated on a 4% polyacrylamide gel.

**Figure 4. Docetaxel enhances the stability of COX-2 mRNA.** Cells were treated with vehicle (A) or 10 μM docetaxel (B) for 3 h. Subsequently, cells received actinomycin D (5 μg/ml) for 0, 30, 60 and 120 min (lanes 1-4, panel A) or 0, 30, 60, and 180 min (lanes 1-4, panel B). Total cellular RNA was isolated. The decay of COX-2 mRNA was analyzed by Northern blotting. The blots shown are representative of three independent experiments. C, the results of three independent experiments for vehicle (control), 10 μM docetaxel and 10 μM paclitaxel-treated cells were quantified. Band density was quantified with a scanning densitometer. Amounts of COX-2 mRNA are expressed as a relative percentage prior to addition of actinomycin D. Bars, SD.

**Figure 5. The AU-rich element of the COX-2 3'-UTR is important for docetaxel-mediated stabilization of COX-2 mRNA.** A, various deletions of the 1455-nucleotide COX-2 3'-UTR (open bars) were fused to the reporter gene luciferase (black bars) to create expression constructs containing the luciferase cDNA fused to the full-length COX-2 3'-UTR (Luc+3'UTR), the COX-2 AU-rich element (Luc+ARE), the AU-rich element deleted
from the full-length 3'-UTR (Luc\^ARE), or luciferase without a 3'-UTR (Luc\^ 3'UTR). The filled circles represent AU-rich sequences, AUUUA, contained within the 3'-UTR. B, 184B5/HER cells were transfected with 1.8 µg of a series of human COX-2 3' UTR deletion constructs ligated to luciferase (Luc+3'UTR, open bar; Luc+ARE, black bar; and Luc \^ARE, speckled bar) and 0.2 µg pSV\(\beta\)gal. After transfection, cells were treated with vehicle (control) or 10 µM docetaxel. Reporter activities were measured in cellular extract 7 h later. Luciferase activity represents data that have been normalized with \(\beta\)-galactosidase. Columns, means; bars, SD; n=6. C, Northern blot analysis. Total cellular RNA was isolated from cells transfected with Luc+3'UTR (lanes 1 and 2) or Luc\^ARE (lanes 3 and 4) and treated with vehicle (lanes 1 and 3) or 10 µM docetaxel (lanes 2 and 4) for 7 h. The blot was hybridized with probes that recognized luciferase mRNA and 18S rRNA. D, 5 µg of cytoplasmic lysate protein was incubated with a \(^{32}\)P-labeled \textit{in vitro} transcribed oligonucleotide containing the ARE of COX-2. Lane 1 represents vehicle (control) treated cells; lanes 2 and 3 represent cytosolic lysates from cells treated for 3 h with 5 µM and 10 µM docetaxel, respectively. E, Cytoplasmic lysate protein was incubated with a \(^{32}\)P-labeled \textit{in vitro} transcribed oligonucleotide containing the ARE of COX-2 following treatment with vehicle (lane 1) or 10 µM docetaxel (lane 2). Lanes 3-6 represent a cold chase experiment in which cytoplasmic lysate protein from cells treated with 10 µM docetaxel was incubated with a \(^{32}\)P-labeled \textit{in vitro} transcribed oligonucleotide containing the ARE of COX-2 and a 50-100-fold excess of unlabeled oligonucleotide containing mutant ARE (lane 3), a non-ARE region of the 3'UTR (lane 4, 300-390 bp) or the ARE (lane 5 (50X), lane 6 (100X)). In lanes 7 and 8, cytoplasmic lysate protein was incubated with a \(^{32}\)P-labeled \textit{in vitro} transcribed oligonucleotide containing mutant ARE of COX-2 following treatment with...
vehicle (lane 7) or 10 μM docetaxel (lane 8). In lanes 9-16, 32P-labeled in vitro transcribed oligonucleotides from different regions of the COX-2 3’UTR in close proximity to the ARE were incubated with cytoplasmic extracts from vehicle treated cells (lanes 9, 11, 13 and 15) or cells treated with 10 μM docetaxel (lanes 10, 12, 14 and 16). In lanes 9-16, the following oligonucleotides from the COX-2 3’UTR were used for binding studies: 400-500 bp (lanes 9, 10), 500-600 bp (lanes 11, 12), 700-800 bp (lanes 13,14), and 900-1000 bp (lanes 15,16). All treatments were for 6 h. In lanes 1-16, 5 μg of cytoplasmic lysate protein was used for binding studies. In D and E, RNA-protein complexes were subjected to electrophoresis as described in Experimental Procedures.

**Figure 6. Docetaxel stabilizes COX-2 mRNA via HuR.** A, 5 μg of cytosolic lysate protein from 184B5/HER cells was incubated with a 32P-labeled in vitro transcribed oligonucleotide containing the ARE of COX-2. Lane 1 represents cytosolic lysate from cells treated with 10 μM docetaxel; lanes 2 and 3 represent cytosolic lysate from docetaxel-treated cells incubated with 1 and 2 μl of anti-HuR antibody, respectively. B, 5 μg of cytosolic lysate protein from 184B5/HER cells was incubated with a 32P-labeled in vitro transcribed oligonucleotide. Lane 1 represents cytosolic lysate from cells treated with 10 μM docetaxel; lanes 2 and 3 represent cytosolic lysate from docetaxel-treated cells incubated with 2 μl of anti-HuR antibody (lane 2) or 2 μl of IgG (lane 3). In lanes 1-3, cytosolic lysate protein was incubated with a 32P-labeled in vitro transcribed oligonucleotide containing the ARE of COX-2. Lanes 4 and 5 represent cytosolic lysate from docetaxel-treated cells incubated with a 32P-labeled in vitro transcribed oligonucleotide containing a non-ARE region of the 3’UTR (300-390 bp). In lane 5 the
lysate was incubated with 2 µl of anti-HuR antibody. In A and B, RNA-protein complexes were subjected to electrophoresis as described in Experimental Procedures. C, In lanes 2-4, cells were transfected with 0.9 µg of Luc+3’UTR construct and 0.2 µg of pSVβgal. In lane 4, cells also received 0.9 µg of antisense (AS) HuR. The total amount of DNA in each of the three reactions was kept constant at 2 µg by using corresponding empty expression vector. Following transfection, cells were treated with vehicle (lane 2) or 10 µM docetaxel for 8 h (lanes 3, 4). Lane 1 represents a standard for HuR. Cellular lysate protein (100 µg/lane) was loaded onto a 10% SDS polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibodies to HuR and β-actin. D, cells were transfected with 0.9 µg of a human COX-2 3’UTR (Luc+3’UTR) construct ligated to luciferase and 0.2 µg of pSVβgal. Bar labeled AS HuR represent cells that received 0.9 µg of antisense (AS) HuR. The total amount of DNA in each reaction was kept constant at 2 µg by using corresponding empty expression vectors. Cells were treated with vehicle (control) or 10 µM docetaxel for 8 h. Luciferase activity represents data that have been normalized to β-galactosidase activity. Columns, means; bars, S.D.; n=6. E, total cellular RNA was isolated from cells transfected and treated as described in panel D. Lane 1, vehicle; lane 2 docetaxel; lane 3, docetaxel plus antisense HuR. 10 µg of RNA was added to each lane. The blot was hybridized with probes that recognized luciferase mRNA and 18S rRNA.

**Figure 7.** PKC signaling is important for both docetaxel-mediated induction of COX-2 transcription and stabilization of COX-2 mRNA. A, cells were treated with 0-10 µM docetaxel for 6 h. Total PKC activity was measured. Columns, means; bar; S.D.
n=4. B, cells were transfected with 0.9 µg of a human COX-2 5’-UTR construct ligated to luciferase (-327/+59; open bars) or 0.9 µg of a human COX-2 3’UTR construct ligated to luciferase (Luc+3’UTR; black bars) and 0.2 µg of pSVβgal. Bars labeled PKC-α WT and PKC-α DN represent cells that received 0.9 µg of expression vector for wild-type or dominant negative PKC-α. The total amount of DNA in each reaction was kept constant at 2 µg by using corresponding empty expression vector. Cells were treated with vehicle (control) or 10 µM docetaxel for 8 h. Luciferase activity represents data that have been normalized to β-galactosidase activity. Columns, means; bars, S.D.; n=6. C, total cellular RNA was isolated from cells transfected with Luc+3’UTR and treated as described in panel B. Lane 1, control; lane 2 docetaxel; lane 3, docetaxel plus PKC-α WT; lane 4, docetaxel plus PKC-α DN. 10 µg of RNA was added to each lane. The blot was hybridized with probes that recognized luciferase mRNA and 18S rRNA.

Figure 8. Docetaxel-mediated induction of COX-2 is dependent on MAP kinases. In panels A-C and F-H, the levels of phosphorylated and unphosphorylated forms of ERK1/2 (A, F), p38 (B, G) and c-Jun (C, H) were measured following treatment for 30 min. A, Phospho ERK 1 and unphosphorylated ERK1/2 MAPK levels were measured in cells treated with vehicle (lane 2) or docetaxel (2.5, 5, 10 µM; lanes 3-5). Lane 1 represents phospho ERK1 standard. B, Phospho-p38 and p38 MAPK levels were measured in cells treated with vehicle (lane 2) or docetaxel (2.5, 5, 10 µM; lanes 3-5). Lane 1 represents phospho-p38 standard. C, JNK activity was determined by measuring the levels of phospho-c-Jun and c-Jun in cells treated with vehicle (lane 1) or docetaxel (2.5, 5, 10 µM; lanes 2-4). Lane 5 represents a phospho-c-Jun standard. D, cells were treated with vehicle
(lane 1), 10 µM docetaxel (lane 2), 10 µM docetaxel plus PD 98059 (25, 50, 100 µM; lanes 3-5, respectively) or 10 µM docetaxel plus SB 202190 (1, 5, 10 µM; lanes 6-8, respectively) for 4.5 h. E, cells were treated with vehicle (lane 2), 10 µM docetaxel (lane 3) or 10 µM docetaxel plus PD 98059 (1 µM; lane 4) for 4.5 h. Lane 1 represents an ovine COX-2 standard. F, cells were treated with vehicle (lane 2), 10 µM docetaxel (lane 3) or 10 µM docetaxel plus PD 98059 (25, 50, 100 µM; lanes 4-6, respectively). Lane 1 represents a phospho-ERK1 standard. G, cells were treated with vehicle (lane 2), 10 µM docetaxel (lane 3) or 10 µM docetaxel plus SB 202190 (1, 5, 10 µM; lanes 4-6, respectively). Lane 1 represents a phospho-p38 standard. H, cells were treated with vehicle (lane 2), 10 µM docetaxel (lane 3) or 10 µM docetaxel plus SB 202190 (0.5 and 1 µM; lanes 4 and 5). Lane 1 represents a phospho-c-Jun standard. In A-H, cell lysate protein was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblots were probed for phospho-ERK1 and ERK1/2 (A, F), phospho-p38 and p38 (B, G) and phospho-c-Jun and c-Jun (C, H). Immunoblots in panels D and E were probed for COX-2.

Figure 9. MAP kinases are important for both transcriptional and post-transcriptional activation of COX-2. Cells were transfected with 0.9 µg of human COX-2 5’-UTR construct ligated to luciferase (-327/+59; panel A) or 3’UTR construct (Luc+3’UTR; panel B) and 0.2 µg of pSVβ-gal. In panels A and B, bars labeled ERK1 DN, JNK DN, p38 DN represent cells that received 0.9 µg of expression vector for ERK1 DN, JNK DN, and p38 DN, respectively. The total amount of DNA in each reaction was kept constant at 2 µg by using corresponding empty expression vectors. In panels A and B, cells were treated with
vehicle (empty bar) or docetaxel (10 µM; black bars) for 8 h. Luciferase activity represents data that have been normalized to β-galactosidase activity. Columns, means; bars, S.D.; n = 6. *, P<0.01; **, P<0.05. C, total cellular RNA was isolated from cells transfected and treated as described in panel B. Lane 1, control; lane 2, docetaxel; lane 3, docetaxel plus ERK1 DN; lane 4, docetaxel plus JNK DN; lane 5, docetaxel plus p38 DN. 10 µg of RNA was added to each lane. The blot was hybridized with probes that recognized luciferase mRNA and 18S rRNA.

Figure 10. MAPKAPK-2 (MK-2) is important for docetaxel-mediated induction of COX-2 mRNA stability. A, MK-2 activity was measured in cells treated with vehicle (lane 1) or docetaxel (5 µM, 10 µM; lanes 2, 3). Cell lysate protein (100 µg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. Immunoblot was probed for phospho-MK-2. B, Cells were transfected with 0.9 µg of human COX-2 3’UTR construct and 0.2 µg of pSVβ-gal. Bar labeled MAPKAKK-2 represents cells that received 0.9 µg of expression vector for wild type MAPKAPK-2 and the bar labeled as MAPKAKK-2 DN represents cells that received 0.9 µg of expression vector for dominant negative MAPKAPK-2. The total amount of DNA in each reaction was kept constant at 2 µg by using corresponding empty expression vectors. Cells were treated with vehicle (Control) or docetaxel (10 µM) for 8 h. Luciferase activity represents data that have been normalized to β-galactosidase activity. Columns, means; bars, S.D.; n = 6. C, total cellular RNA was isolated from cells transfected and treated as described in panel B. Lane 1, control; lane 2, docetaxel; lane 3, docetaxel plus MAPKAPK-2 WT; lane 4, docetaxel plus MAPKAPK-2 DN. 10 µg of RNA was added to
The blot was hybridized with probes that recognized luciferase mRNA and 18S rRNA.

**Figure 11. Treatment with a p38 MAPK inhibitor or dexamethasone suppresses docetaxel-mediated stabilization of COX-2 mRNA by inhibiting HuR binding to COX-2 ARE.** A, Cells were transfected with 1.8 µg of human COX-2 3’UTR construct ligated to luciferase (Luc+3’UTR) and 0.2 µg of pSVβgal. Cells were treated with vehicle (control), docetaxel (10 µM), docetaxel plus 10 µM SB 202190 or docetaxel plus 1 µM dexamethasone for 8 h. Luciferase activity represents data that have been normalized to β-galactosidase activity. Columns, means; bars, S.D.; n = 6. B, total cellular RNA was isolated from cells transfected and treated as described in panel A. Lane 1, control; lane 2, docetaxel; lane 3, docetaxel plus SB 202190; lane 4, docetaxel plus dexamethasone. 10 µg of RNA was added to each lane. The blot was hybridized with probes that recognized luciferase mRNA and 18S rRNA. C, 5 µg of cytoplasmic lysate protein from cells was incubated with a 32P-labeled *in vitro* transcribed oligonucleotide containing the ARE of COX-2. Lane 1 represents vehicle; lanes 2-5 represent cytosolic lysates from cells treated with docetaxel (10 µM; lane 2), docetaxel plus 100 µM PD 98059 (lane 3), docetaxel plus 10 µM SB 202190 (lanes 4) or docetaxel plus 1 µM dexamethasone (lane 5). RNA-protein complexes were subjected to electrophoresis as described in *Experimental Procedures*.

**Figure 12. Nocodazole stimulates the COX-2 3’-UTR via the AU-rich element.**
A, 5 \mu g of cytoplasmic lysate protein from cells was incubated with a $^{32}$P-labeled in vitro transcribed oligonucleotide containing the ARE of COX-2. Lane 1 represents cytosolic lysates from vehicle-treated cells; lanes 2 represents cytosolic lysates from cells treated with 10 \mu M nocodazole for 6 h. RNA-protein complexes were subjected to electrophoresis as described in Experimental Procedures. In B and C, cells were transfected with 1.8 \mu g of a series of human COX-2 3′ UTR deletion constructs ligated to luciferase (Luc+3′UTR, open bar; Luc+ARE, black bar; and Luc▲ARE, speckled bar) and 0.2 \mu g pSV-\beta gal. After transfection, cells were treated with vehicle (control) or 10 \mu M nocodazole. Reporter activities were measured in cellular extract 7 h later. Luciferase activity represents data that have been normalized with \beta-galactosidase (panel B). Columns, means; bars, SD; n=6. In C, total cellular RNA was isolated from transfected cells (lanes 1 and 4, Luc+3′UTR; lanes 2 and 5, Luc+ARE; lanes 3 and 6, Luc▲ARE). Lanes 1-3 represent cells that were treated with vehicle. Lanes 4-6 represent cells that were treated with 10 \mu M nocodazole. 10 \mu g of RNA was added to each lane. The blot was hybridized with probes that recognized luciferase mRNA and 18S rRNA.

Figure 13. Taxanes induce COX-2 expression by both transcriptional and post-transcriptional mechanisms. Stimulation of protein kinase C (PKC) by taxanes enhances mitogen activated protein kinase (MAPK) activity, which results, in turn, in AP-1-mediated induction of COX-2 transcription. Taxanes also induce COX-2 by stabilizing COX-2 mRNA. A signaling pathway that includes PKC\rightarrow p38 MAPK\rightarrow MAPKAPK-2 (MK-2) is responsible for the increase in COX-2 message stability following taxane treatment. The 3′-untranslated region (UTR) of COX-2 mRNA contains a series of sequences (AUUUA)
known as AU-enriched elements (ARE) that confer message instability. Augmented binding of HuR, an RNA binding protein, to the ARE of the COX-2 3′-UTR is responsible at least, in part, for the observed increase in COX-2 message stability following taxane treatment.
Figure 1

A

![Bar graph showing PGE2 levels in response to different concentrations of Docetaxel.](image)

B

![Western blot for COX-2 expression.](image)

C

![Western blot for COX-2 and 18S rRNA expression.](image)

D

![Western blot for COX-2 and 18S rRNA expression.](image)
Figure 2

A

-1432  
-223/-214  
-132/-124  
-59/-53  
-31/-25 (TATA Box)  

NFκB  
NF-IL6  
CRE  

B

Luciferase Activity

Control  
Docetaxel  

-1432  
-327  
-220  
-124  
-52  

C

Luciferase Activity

-327/ +59  
KBM  
ILM  
CRM  

Control  
Docetaxel
Figure 8

A

Phospho-ERK-1

ERK1/2

1 2 3 4 5

B

Phospho-p38

p38

1 2 3 4 5

C

Phospho-c-Jun

c-Jun

1 2 3 4 5

D

COX-2

1 2 3 4 5 6 7 8

E

COX-2

1 2 3 4

F

Phospho-ERK1

ERK1/2

1 2 3 4 5 6

G

Phospho-p38

p38

1 2 3 4 5 6

H

Phospho-c-Jun

c-Jun

1 2 3 4 5
Figure 9

A

Luciferase Activity

-327/+59 -327/+59 -327/+59 -327/+59
+ ERK1 DN + JNK DN + p38 DN

B

Luciferase Activity

3'UTR 3'UTR 3'UTR 3'UTR
+ ERK1 DN + JNK DN + p38 DN

C

Luciferase 18S rRNA

1 2 3 4 5
Figure 11

A

![Bar chart showing luciferase activity with COX-2 3'UTR](chart.png)

B

![Western blot of Luciferase and 18S rRNA](blot.png)

C

![Gel electrophoresis](gel.png)
Regulation of cyclooxygenase-2 mRNA stability by taxanes. Evidence for involvement of p38, MAPKAPK-2 and HuR®
Kotha Subbaramaiah, Timothy P. Marmao, Dan A. Dixon and Andrew J. Dannenberg

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