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GRANT NUMBER DAMD17-94-J-4259

TITLE: The Role of Prostaglandins in the Growth of Breast Epithelial Cells

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REPORT DATE: September 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | September 1998 | Final (14 Aug 94 - 15 Aug 98) |

**4. TITLE AND SUBTITLE**
The Role of Prostaglandins in the Growth of Breast Epithelial Cells

**5. FUNDING NUMBERS**
DAMD17-94-J-4259

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**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

**10. SPONSORING/MONITORING AGENCY REPORT NUMBER**



**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200*

Dietary fatty acids have been implicated as risk factors in breast carcinogenesis and our work, as well as that of others, has shown that they are regulators of the growth of breast epithelial cells. This project studied how linoleic acid and its metabolites stimulate the growth of breast epithelial cells. We found that this fatty acid causes induction of cyclooxygenase-2 by increasing transcription, and that this, in turn, generates products that stimulate growth. Linoleic acid and the products exert these action by binding to a target receptor, PPAR, which serves as a transcription factor that binds to an element in the promoter of the COX-2 gene, a mechanism identified for the first time in this project. We also discovered that the level of expression of COX-2 is controlled by the efficiency of translation of the mRNA and have shown that several proteins specifically mediate the effect. In summary, this project has determined the mechanism by which fatty acids stimulate the proliferation of breast cells, a process that offers the potential for intervention either by dietary manipulation or by chemoprevention using inhibitors of COX-2.

**14. SUBJECT TERMS**
Breast Cancer

**15. NUMBER OF PAGES**
19

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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Stephen M. Prescott Sept. 10, 1998

PI - Signature Date

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## Introduction

*Nature of the problem*

Prostaglandins and related compounds are generated by the addition of molecular oxygen to polyunsaturated fatty acids in reactions catalyzed by specific oxygenases. The committed step in the synthesis of prostaglandins is prostaglandin G/H synthase (also known as cyclooxygenase; PHS, COX), of which there are two forms. One is thought to play a key role in carcinogenesis—particularly in the colon (1), but potentially in other tissues as well (2). Prostaglandins mediate many cellular responses, including growth, differentiation and apoptosis. They have been implicated in mouse studies of mammary carcinogenesis as well as epidemiological studies in humans (3-6). This is intriguing because it may be the link between dietary fat content and an increased risk of breast cancer. In work reported in the first three years of the project, we demonstrated that major dietary fatty acids stimulate the growth of mammary epithelial cells and that this response requires the induction of COX-2. We showed that PPARs mediate the fatty acid effects on transcription of COX-2, and we described a novel regulatory mechanism for post-transcriptional regulation.

*Background*

A typical response to various stimuli is for cells to activate phospholipases and then use the resulting free arachidonic acid to synthesize prostaglandins and related compounds, which are collectively known as eicosanoids (7). Linoleic acid, which is a precursor of arachidonic acid, also can be converted to related oxidized compounds directly; theses are termed HODEs. Eicosanoids elicit effects locally, often stimulating the tissue production, and these effects are exerted by binding to specific receptors on the cell surface—a family of G-protein coupled receptors. Alternatively, they can bind to a small family of nuclear receptors, the PPARs, and exert transcriptional activation of target genes (8-10). Eicosanoids are second messengers in many physiological and pathological responses including regulation of growth and carcinogenesis. The key step in the conversion of free arachidonic acid to prostaglandins is catalyzed by prostaglandin H synthase (PHS). This enzyme has been previously known as cyclooxygenase and is the main target for inhibition by aspirin and non-steroidal anti-inflammatory drugs.

The total activity of PHS varies markedly in response to cytokines, growth factors, tumor promoters, and other stimuli, and the key change now is known to be the induction of a second isoform, which is an early inducible gene (1, 11, 12). Much of the induction is at the transcriptional level, but we also have examined whether downstream events that target the 3' untranslated region (3'UTR) of the human COX-2 mRNA influence the level of protein. The 3'UTR contains multiple AUUUA repeats, and this sequence is found in the 3'UTRs of many immediate-early genes including proto-oncogenes. The general view is that these sequences influence expression by making the mRNA unstable (13). However, as we showed last year, an alternative explanation, altered translational efficiency, seems to be the major mechanism for the effects on COX-2 expression.

*Purpose of the present work*

We have four specific aims:

1. Determine the metabolic fate of linoleic acid that stimulates the growth of breast epithelial cells
2. Determine whether prostaglandin H synthase (cyclooxygenase) is induced during breast tumorigenesis
3. Test the hypothesis that metabolites of arachidonic acid are essential mediators of the responses of breast epithelial cells to EGF and phorbol esters
4. Determine the molecular basis for regulation of expression of PHS2 (COX-2)

*Note: the original proposal had a fifth aim but it was not recommended for funding in the original review and budgetary changes were made to accommodate that criticism. Thus, as recommended, we have not pursued it.*

*Methods of approach*

***Reporter constructs.*** Regions of the 7 kb COX-2 promoter were cloned into pGL3-basic (Promega). The putative PPRE in the COX-2 promoter (located at -3900), as well as a consensus PPRE for the rat acyl CoA oxidase gene (Tugwood, Marcus) were cloned into pGL3-promoter. Oligonucleotide sequences for ACOX PPRE were:

ACOX PPRE F - 5' CGCGT TCCTTTCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCTA 3',

ACOX PPRE R - 5' GATCTAGCAAAAGGGGACCAGGACAAAGGTCACGTTCGGAAAGGA 3',

and for the COX-2 -3900 PPRE:

COX-2 -3900 PPRE F - 5' CGCGTGGTCTGTCTTTCAAATTTTTTAAGTAGGGTTATGACCTG TCGCCTCACTTCTCTGACAGTTCTA 3',

COX-2 -3900 PPRE R - 5' GATCTAGAACTGTCAGAGAAGTGAGGCGACAGGTCATAACCC TACTTAAAAAATTTGAAAGACAGACCA 3'.

pGL3-promoter with no insert was used as a negative control in transfection experiments. PPARα cloned into pcDNAI was used for cotransfection to ensure that the cells' native PPAR would not be limiting in detection of transcriptional effects. β-galactosidase cloned into pHOOK-2 (Invitrogen) was used as a control for normalization of transfections.

***Epithelial cell culture.*** 184B5 mammary epithelial cells (obtained from Martha Stampfer) were grown in modified MCDB 170, a serum-free defined medium. Prior to treatment with fatty acids, prostaglandins, or NSAIDs, the cells were made quiescent by treating for 48 hr with an antibody to the epidermal growth factor receptor (MAb 225) at 10 μg/ml. To examine COX-2 expression in mammary epithelial cells, medium was changed to fresh serum-free medium containing the fatty acid or prostaglandin. For 184B5 cells, the medium also contained the anti-EGF receptor antibody. Cells were incubated with agonist for 4-8 hr for mRNA expression, or for 10-12 hr, for protein expression, prior to harvest. For time course experiments, samples were harvested at the times indicated in the figures. For each fatty acid, prostaglandin and NSAID used, a dose-response curve was performed. Prostaglandins, HETEs and HODEs were used at the following concentrations: $PGD_2$, $PGE_2$, and $PGF_{2\alpha}$ at 0.4-74 μM, $PGA_2$, 15-deoxy-$\Delta^{12,14}$-$PGJ_2$, 8(R)-HETE, 8(S)-HETE, 15-HETE, 9-HODE and 13-HODE at 0.3-18 μM.

***Transfection experiments.*** Quiescent 184B5 cells were transfected using 2.5 μl/well LipofectAmine (Gibco-BRL) and 0.25 μg/well each of luciferase reporter plasmid,

PPARα, and β-galactosidase, and were performed in the presence of MAb 225. Twenty-four hr following transfection, medium was changed to fresh MCDB 170 containing MAb 225 with or without 100 μM WY-14,643. Seventy-two hr later, the cells were harvested, and luciferase and β-galactosidase assays were performed. Luciferase activity was measured using Promega's luciferase assay kit, and β-galactosidase was measured using Galactolight (Tropix). Triplicate wells were assayed in duplicate for each sample.

***Western blot analysis.*** Cells were washed with ice-cold PBS, then lysed with buffer consisting of 20 mM Tris, pH 7.5, 16 mM CHAPS, 0.5 mM DTT, 1 mM EDTA, 1 mM benzamidine hydrochloride, 1 μg/ml leupeptin and 10 μg/ml soybean trypsin inhibitor. Cells were scraped and placed on ice for 30 min. Unlysed cells and debris were removed by centrifugation, and protein assays were performed (BCA protein assay, Pierce) to ensure equal loading on SDS-PAGE. Proteins were separated through 10% denaturing polyacrylamide gels and transferred to PVDF membranes. COX-2 and COX-1 monoclonal antibodies were used according to established procedures (16). Monoclonal anti human β-actin was also used in some experiments as a control for protein integrity. Secondary antibody was HRP-labeled goat anti-mouse (Biosource). ECL (Amersham) was used to visualize Westerns. Quantitation of Western blots was performed by scanning the blots into Photoshop and densitometric quantitation using NIH Image.

***RNase protection assay.*** RNA was isolated using TRIzol reagent (Gibco-BRL) and processed according to the manufacturer's instructions. Samples were resuspended in RNase-free water and concentrations determined spectrophotometrically. Ten μg of each sample was used for RNase protection assay with probes for COX-2 and GAPDH. The COX-2 probe corresponds to the region spanning -90 to +332 relative to the COX-2 translational start site (15). The GAPDH probe is commercially available (Ambion). Radioactive transcripts were prepared using [$^{\alpha 32}$P]-UTP and Ambion's MAXIscipt *in vitro* transcription kit. Eighty-thousand cpm/sample of the COX-2 probe and 8000 cpm/sample of the GAPDH probe were used in the RNase protection assay (RPA II - Ambion). RNA and probes were co-precipitated, resuspended in hybridization buffer and incubated overnight at 43°. The following day, unbound RNA was digested with RNase, the remaining radiolabeled RNA was precipitated, resuspended in loading buffer and separated on a 6% polyacrylamide gel. Gels were dried and exposed to BioMax MS film at -80° for up to 48 hr. Following autoradiography, the protection assay was quantitated as described above.

***Gel shift assay.*** Nuclear extracts were prepared from Cos-7 cells transfected with PPARδ and RXRα (Andrews and Faller). Oligonucleotide cassettes corresponding to the ACOX PPRE (Tugwood, Marcus) and the COX-2 -3900 PPRE were either radiolabeled using [$\gamma^{32}$-P]-ATP and T4 kinase, or used unlabeled for competition experiments. The following oligonucleotides were used:

ACOX PPRE F - 5' TCCTTTCCGAACGTGACCTTTGTCCTGGTCCCCTTTTGCTA 3',
ACOX PPRE R - 5' TAGCAAAAGGGGACCAGGACAAAGGTCACGTTCGGAAAGGA 3',
COX-2 -3900 PPRE F - 5' GGTCTGTCTTTCAAATTTTTTAAGTAGGGTTATGACCTGTCGC CTCACTTCTCTGACAGTTCT 3',
COX-2 -3900 PPRE R - 5' AGAACTGTCAGAGAAGTGAGGCGACAGGTCATAACCCTACT TAAAAAATTTGAAAGACAGACC 3',
AP2 F - 5'GATCGAACTGACCGCCCGCGGCCCGT 3'

AP2 R - 5' ACGGGCCGCGGGCGGTCAGTTCGATC 3'.
Binding reactions were performed by preincubating 20 µg of nuclear lysate in binding buffer with or without competitor oligonucleotide (fifty-fold excess compared to hot oligonucleotide) in a volume of 18 µl for 10 min at room temperature. Binding buffer consisted of 10% glycerol, 20 mM Tris, pH 8.0, 80 mM KCl, 10 mM $MgCl_2$, 2 mM DTT and 5 µg poly dI·dC. Two µl of radiolabeled oligonucleotide cassette was then added and the reactions incubated 20 min at room temperature. Binding reactions were separated by loading on a prerun 4% non-denaturing PAGE containing 2.5% glycerol and separated in 1xTBE buffer at 150V at room temperature until the bromophenol blue marker dye was approximately 2/3 of the way down the gel. Controls, including AP2 oligonucleotides, were from Promega's Gel Shift Assay System. Gels were dried and exposed to BioMax MR autoradiography film at -80° for up to 48 hr.

## Body

**Aim # 1. Determine the metabolic fate of linoleic acid that stimulates the growth of breast epithelial cells.**
and
**Aim #3. Test the hypothesis that metabolites of arachidonic acid are essential mediators of the responses of breast epithelial cells to EGF and phorbol esters.**
In previous reports we submitted evidence that fatty acids induce the expression of COX-2 and that this is the mechanism for growth; *i.e.* the growth response was blocked by a specific COX-2 inhibitor. Thus, as described last year, the first aim was completed but had become closely related to the third aim. Thus, they will be considered jointly in this report—our focus for the last year has been to define the role of prostaglandins and PPARs in the COX-2 induction. The pattern of fatty acids which enhanced COX-2 expression is consistent with fatty acids that have recently been identified to act through the steroid hormone receptors known as peroxisome proliferator activated receptors (PPARs). PPARs are orphan members of the steroid hormone receptor superfamily. While their true ligands are not known, it has been demonstrated that many prostaglandins, as well as fatty acids can act as ligands. We tested the ability of a panel of potential COX-2 products to enhance its expression. We found that many of the fatty acids that we tested were capable of enhancing COX-2 expression, particularly the cyclopentanone $PGA_2$ and $15dPGJ_2$, which are most potent in other PPAR-mediated responses (Figure 1a). Of all the compounds that we tested, $15dPGJ_2$ was the most potent, strongly enhancing COX-2 expression at levels as low as 0.4 µM (Figure 1b). We also found that 15-HETE (Figure 1a), 8-HETE and 9- and 13-HODE stimulated COX-2 expression (not shown). Somewhat surprisingly, $PGE_2$, which is produced at relatively high levels by mammary epithelial cells, did not alter COX-2 expression in these cells. This pattern of COX-2 expression in response to prostaglandins and HETEs supported the hypothesis that it occurred via a PPAR. As an additional test of this, we treated cells with the PPAR-selective agonist WY-14,643 and found that it strongly stimulated COX-2 expression (Figure 1c).

Peroxisome proliferators enhance expression of the genes that they regulate transcriptionally. If fatty acids, prostaglandins and NSAIDs alter COX-2 expression through a PPAR, changes in COX-2 mRNA levels should correspond with changes in COX-2 protein levels. We examined the changes in COX-2 mRNA over time in 184B5 cells treated with linoleic acid and found that linoleic acid strongly enhanced COX-2 expression, reaching a maximum by 4 hr following treatment. We further tested the role that transcription plays in peroxisome proliferator enhanced COX-2 expression by examining the effects of the transcriptional inhibitor actinomycin D on this response. Actinomycin D inhibited COX-2 expression when used in combination with fatty acids, or prostaglandins (not shown), providing further evidence that their effects are transcriptional. Nuclear run-on assays validated this hypothesis; 18 μM LA enhanced COX-2 transcription in 184B5 cells (1.71+/- 0.30) fold (COX-2/COX-1), while PMA, which strongly stimulates COX-2 expression in these cells, enhanced transcription (2.14 +/- 0.38) fold (n=2).

In earlier reports, we showed that all three isoforms of PPAR were present in breast epithelial cells, we cloned all of the human isoforms for PPAR, and made anti-peptide antibodies to each of the isoforms. In the past year we made reporter constructs that contained between 7 kb and 200 bp of the COX-2 promoter, the -3900 PPRE from COX-2 (described last year), and a reporter construct that contained the rat fatty acyl CoA oxidase (ACOX) PPRE sequence (positive control). Cells were co-transfected with an expression plasmid for PPARα. The transfected cells were then stimulated with WY-14,643, or vehicle for 72 hr. Luciferase activity was measured and normalized to cotransfected β-galactosidase. In cells containing the -3900 PPRE construct, luciferase activity increased 1.4-fold on treatment with WY-14,643 (n=6) (Figure 2a). This response depended on cotransfection with PPARα, and, while modest, was highly consistent among experiments. In the second approach, we performed gel shift experiments using nuclear lysate obtained from Cos-7 cells transfected with a PPAR and RXRα (Figure 2b). As a positive control we used an oligonucleotide cassette for the ACOX PPRE. Both the COX-2 -3900 PPRE and the ACOX PPRE produced bands on gel shift. The ACOX PPRE could be competed with cold ACOX PPRE, but not with cold AP2. The COX-2 -3900 PPRE could be competed with cold COX-2 PPRE or ACOX PPRE, but not with cold AP2, supporting the hypothesis that this region of the COX-2 promoter is responsible for binding PPAR/RXR heterodimers, and therefore, responsible for peroxisome proliferator-stimulated transcription of COX-2.

Thus, we have completed all of the planned experiments for these aims, including a detailed mechanistic analysis that was unanticipated at the time of the original application. We have shown that COX-2 is induced by fatty acids and by downstream products, and that this works by the transcription factors, PPARs. The newly expressed COX-2 synthesizes compounds that support increased proliferation of the breast cells. Thus, dietary fatty acids may alter breast responses via this pathway.

**Aim #2. Determine whether prostaglandin H synthase (cyclooxygenase) is induced during breast tumorigenesis.**

In the previous report we described the production of a new, high-affinity antibody with which to carry out immunochemical experiments. Two types of experiments have

been carried out. In the first, we examined a series of breast epithelial cells in culture. The 184 cells were grown originally from a sample of healthy human breast tissue and are normal by many criteria. When tested on multiple occasions for COX-2, there was no staining either by immunocytochemistry or Western blotting. A cell line derived from 184 (184A1) by chemical mutagenesis has been shown to be immortalized but not transformed. Likewise, multiple examinations of this line were negative for COX-2. Another line derived from mutagenesis, 184B5, is also not completely transformed but has some tumor features, including morphological changes. This line consistently had constitutive, although low, expression of COX-2 (not shown). These results on cell lines were consistent with the hypothesis that COX-2 induction is a component of transformation but were not definitive. Our other approach has been to examine samples of human breast tissue for COX-2 expression. This has been plagued with technical difficulties that are largely a reflection of background fluorescence in the tissues after staining. This, of course, also decreases sensitivity as we have to take care to exclude the false positive areas. As we showed last year, there is COX-2 expression in some breast tumors. We now have technically adequate analyses on five tumor samples—four primary and one metastatic to lymph nodes. Of the primary tumors, two were positive for COX-2. The metastatic tumor, which was of the colloid type, was strongly positive. Normal breast epithelial cells (as shown last year) were always negative. Thus, our results support the hypothesis that COX-2 induction is a common, but not universal, manifestation of breast transformation. The high expression in metastatic tumor is intriguing--another group has observed the same in lung cancer and suggested that COX-2 expression may be a mechanistic factor in metastasis (2). Although the DOD funding for this project has ended, we will pursue additional studies of COX-2 expression in these tissues supported by institutional funds.

**Aim #4. Determine the molecular basis for regulation of expression of PHS2.**

In the previous reports we described a novel mechanism for the regulation of expression of COX-2—translational control by the 3'UTR of the mRNA. In those experiments we had found that deletion of the entire 3'UTR, or selected portions, resulted in increased protein expression with what appeared to be the same level of mRNA. Thus, increased translational efficiency seemed to be the most robust explanation. However, the general view of the role of AUUUA sequences is that they confer instability on the mRNA. Thus, we have performed additional experiments examining this point. In addition, it is clear from our work and that of others that increased transcription is an important control mechanism for COX-2 both in inflammation and carcinogenesis (14). One of the key points we proposed originally was to test the effect of steroid hormones on expression. As shown in Figure 3, treatment of cells with dexamethasone alone did not induce transcription of COX-2, as measured by a promoter/reporter construct (see above). The positive control of PMA stimulation gave a marked increase in transcription. Dexamethasone in addition to PMA markedly suppressed COX-2 as judged by the expression of the endogenous gene (Fig.3a) but did not suppress transcription (Fig.3b)—in fact, it appeared to have increased it. This result suggested that post-transcriptional effects could be at work as well. To assess this, we utilized our sensitive RNase protection assay (15) to measure the T1/2 of mRNA after gene induction in the presence or absence of dexamethasone. As shown in Figure 4, the steroid dramatically reduced the

stability of the mRNA. We conclude that the suppressive effects of dexamethasone on COX-2 levels result largely from this mechanism.

Thus, the experiments reported here and in previous reports have shown multiple levels of regulation of COX-2: increased transcription (e.g. through PPARs), regulated translation (last year's report) and steroid-induced changes in mRNA stability. These many levels of control are consistent with this being an important step that needs careful control.

## Conclusions

This project has made several important findings. The first, and most important, is that breast epithelial cells are stimulated to proliferate by unsaturated fatty acids or their downstream metabolites. The pathway involves expression of COX-2, which occurs through increased transcription mediated by PPARs. The level of COX-2 also is regulated by post-transcriptional mechanisms including the rate of translation, which is controlled by a specific set of proteins, and the stability of the mRNA—particularly in the presence of steroid hormones.

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## Appendix

*Figure Legends*

**Figure 1. Peroxisome proliferators enhance COX-2 protein expression in mammary epithelial cells.** A) Products of COX-2 enhance its expression. A dose-response curve was performed for each compound, the concentration shown is that which was maximally effective: 18 μM LA, 18 μM $PGA_2$, 74 μM $PGD_2$, 3.6 μM $15dPGJ_2$, 74 μM $PGF_{2\alpha}$, and 18 μM 15-HETE. Representative of two experiments. B) COX-2 expression is elevated by low levels of $15dPGJ_2$. Western blot analysis of COX-2 expression in response to LA or increasing concentrations of $15dPGJ_2$. Representative of two experiments. C) The prototypical peroxisome proliferator WY-14,643 enhances COX-2 expression in mammary epithelial cells. Western blot analysis of COX-2 expression in response to 18 μM LA, 3.6 μM 15dPGJ2 and WY-14,643. Representative of six experiments.

**Figure 2. Fatty acids stimulate transcription of COX-2 though a PPAR.** A) Peroxisome proliferators stimulate the transcription of COX-2. 184B5 cells were transfected with reporter constructs from the COX-2 promoter, the ACOX PPRE, or a control promoter, driving luciferase expression. Cells were co-transfected with both PPARα and β–galactosidase. Luciferase and β-galactosidase activities were measured 72 hr following the addition of fresh medium with or without 100 μM WY-14,643. Results are expressed as fold increase in luciferase activity on treatment with WY-14,643 (n=6). B) Gel shift analysis. Nuclear lysates were prepared from cos-7 cells transfected with PPARδ and RXRα. Following the binding of oligonucleotide cassettes corresponding to the ACOX PPRE or the COX-2 -3900 PPRE to these lysates, DNA-protein complexes were detected by separation on non-denaturing PAGE. For competition experiments, cold oligonucleotide cassette for ACOX, the COX-2 -3900 PPRE, or AP2 was incubated with the lysates prior to the addition of radiolabeled ACOX or COX-2 PPRE. Representative of two experiments.

**Figure 3. Dexamethasone Can Suppress COX-2 Expression on a Post-Transcriptional level.** A) COX-2 protein expression was induced in HeLa cell monolayers treated for 8 hours with PMA (60ng/ml), or IL-1α (10ng/ml) as detected by Western blot analysis. The addition of Dex (1μM) along with PMA or IL-1α (+Dex lanes) resulted in supression of induced COX-2 protein expression. B) A reporter gene constructed from 2.0 kb of the 5' sequence of the human COX-2 gene and the luciferase gene in the pGL2-Basic vector was cotransfected with the pSV-β-gal control vector into HeLa cells. Tranfected cells were then treated with sham control, PMA (60ng/ml), Dex (1μM), or a combination of PMA and Dex for 12 hours. Cell monolayers were lysed and COX-2 promoter expression was assessed as luciferase activity normalized to control β-galactosidase activity.

**Figure 4. Dexamethasone Induces Rapid Degradation of the COX-2 mRNA.** HeLa cells were stimulated with PMA for 2 hours to induce COX-2 mRNA expression then treated with sham control, ActD (5μg/ml), Dex (1μM), or ActD and Dex together. Total RNA was isolated over the indicated time points and subjected to Rnase protection assay to determine COX-2 and control GAPDH mRNA levels. COX-2 mRNA amounts were normalized to control GAPDH mRNA levels and plotted to determine COX-2 mRNA half-life values shown in the figure legend (n.d. = not detectable)

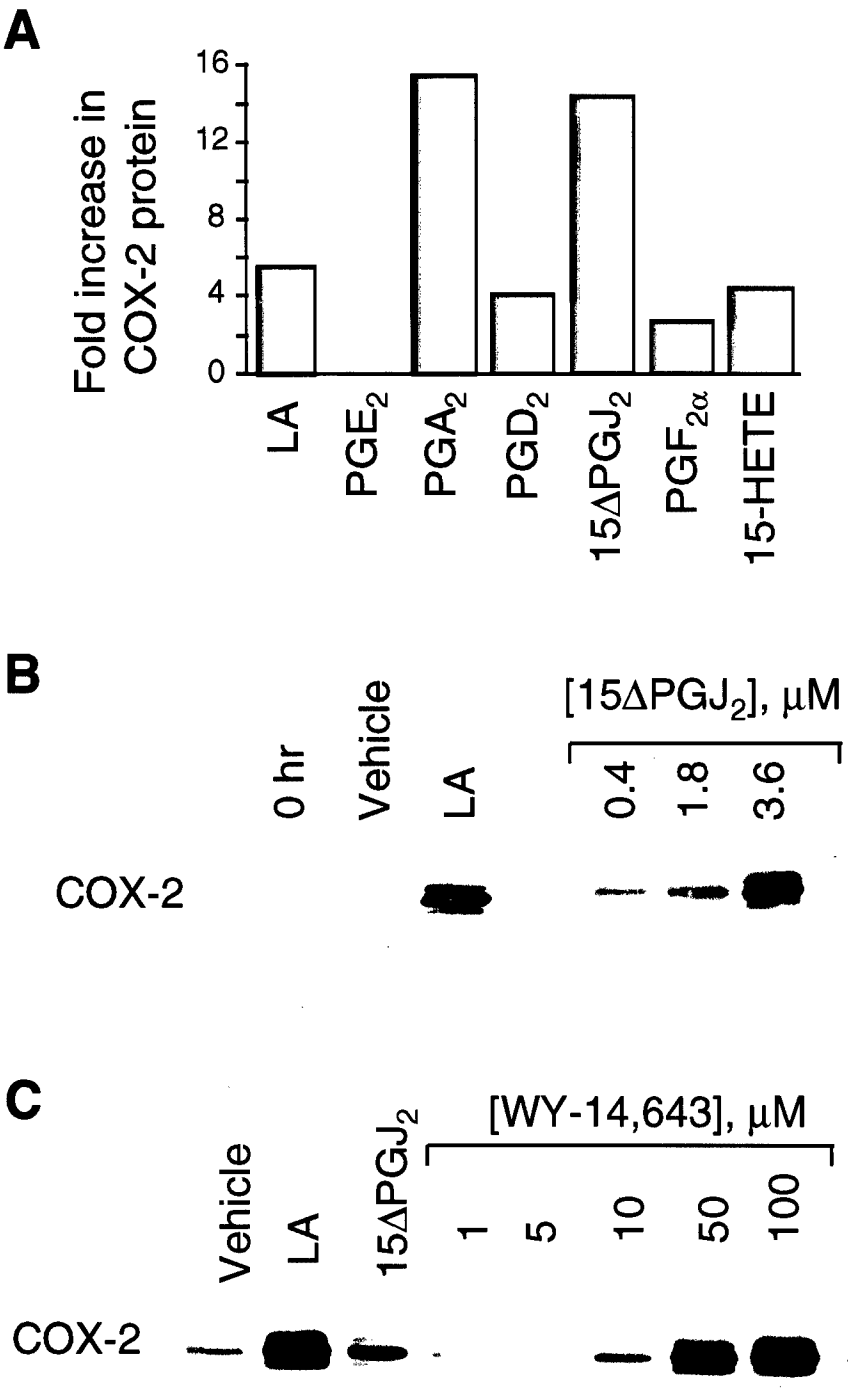




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Fig. 1

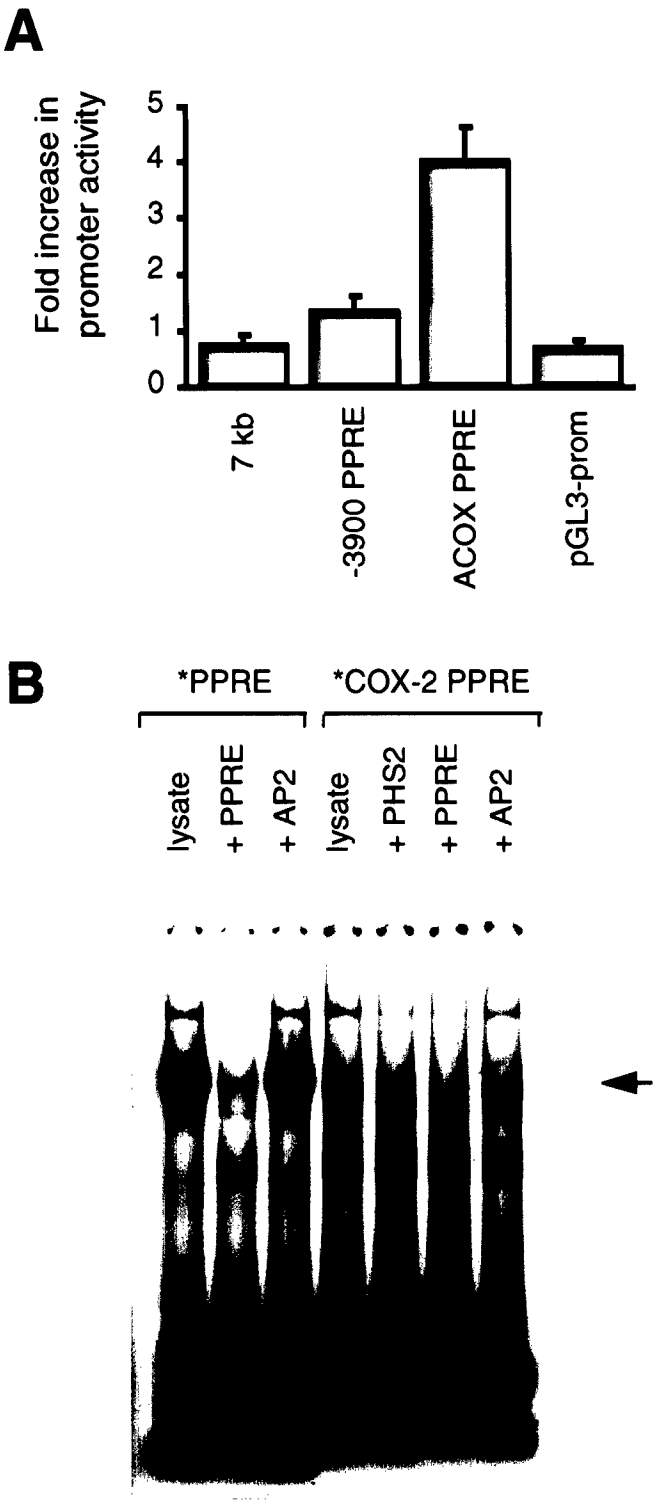




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Fig. 2

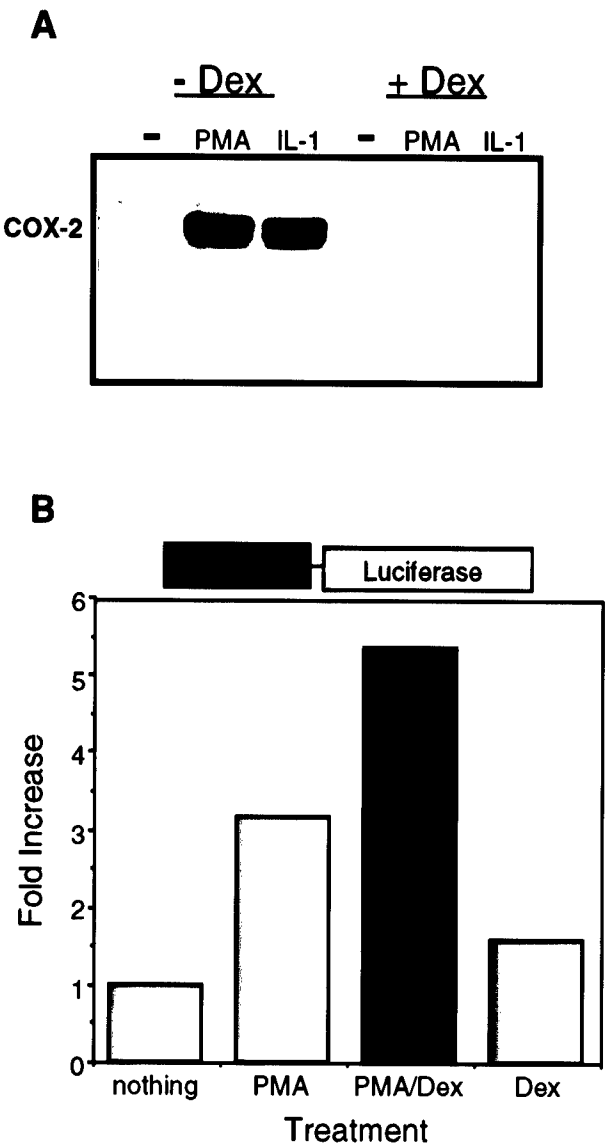




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Fig. 3

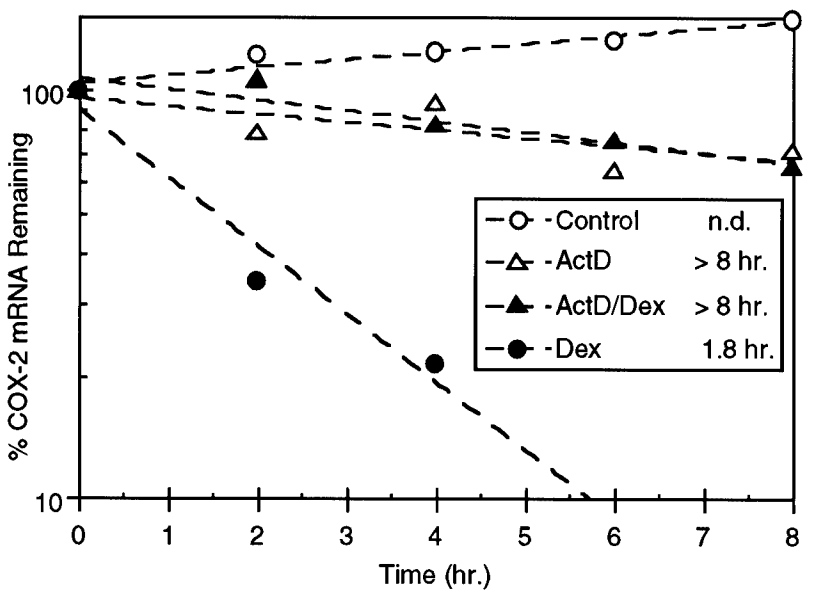




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Fig. 4

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