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TRANSCRIPTIONAL REGULATION OF PROSTAGLANDIN H SYNTHASE (PGHS)-2 GENE IN A MACROPHAGE MODEL OF INFLAMMATION

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William L. Smith
Major Professor's Signature

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TRANSCRIPTIONAL REGULATION OF PROSTAGLANDIN H SYNTHASE (PGHS)-2 GENE IN A MACROPHAGE MODEL OF INFLAMMATION

By

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ABSTRACT

TRANSCRIPTIONAL REGULATION OF PROSTAGLANDIN H SYNTHASE (PGHS)-2 GENE IN A MACROPHAGE MODEL OF INFLAMMATION

By

Yeon-Joo Kang

Prostaglandins, cyclooxygenase (COX, PGHS) products, are lipid-derived hormones that play a crucial role in the development of local and systemic inflammatory responses. Chronic inflammation is an essential step in the progression of many diseases, such as atherosclerosis, cancer, and neurodegenerative diseases. Two COX isozymes, COX-1 and COX-2, are responsible for the production of prostaglandin H_2, the committed step in prostanoid biosynthesis. COX-2 is involved in immune responses. Nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors are commonly used to relieve symptoms associated with inflammation by targeting COX-2.

Exposure of macrophages to lipopolysaccharide (LPS), a major component of the outer membrane of Gram negative bacteria induces sustained COX-2 activation. LPS mediates signaling through the toll-like-receptor-4 (TLR4), which results in the activation of JNK, ERK, and p38 MAP kinase cascades and NF-κB inducing kinase (NIK). I have examined COX-2 gene expression in RAW 264.7 macrophages treated with LPS as a model for COX-2 gene expression during inflammation. Using this system, I have established that COX-2 is highly regulated at the transcriptional level and that LPS-induced COX-2 transcriptional activation is sustained for at least 12 hr after LPS stimulation and occurs in three phases including an initial increase (1-4 hr), a middle

phase (4-9 hr), and a second increase (9-12 hr).

LPS-induced transcriptional activation of COX-2 is mediated by the binding of transcription factors such as CREB, NF-κB, ATF, AP-1, C/EBP to *cis*-acting elements present in the COX-2 promoter. Previous studies with LPS-treated RAW 264.7 cells identified three *cis*-acting elements including a NF-κB site, a C/EBP site, and a CRE (CRE-1). Using promoter analysis, three additional functional *cis*-acting elements in the COX-2 promoter--a second CRE site (CRE-2), an AP-1 site, and an E-box that overlaps the CRE-1 were identified. Five of those elements are involved in COX-2 transcriptional activation, while the E-box appears to be involved in COX-2 transcriptional repression. I also characterized *trans*-acting factors involved in several different phases of sustained activation of COX-2 in LPS-stimulated RAW 264.7 macrophages using electrophoretic mobility supershift assays and chromatin immunoprecipitation assays. I observed that CREB is constitutively bound to CRE-1 or CRE-2 during the entire 12 hr time period. The initial increase in COX-2 transcription involves p65/p50 binding to an NF-κB site and phosphorylated c-Jun/c-fos binding to an AP-1 site. The p65/p50 heterodimer is replaced by p50 homodimers at 4 hr of LPS-treatment and phosphorylated c-Jun/c-fos binding is replaced by phosphorylated c-Jun homodimers after 1 hr. Treatment of cells with JNK and p50 inhibitor after 3.5 hr of LPS stimulation abolished COX-2 mRNA induction at 6 hr; however partial recovery of COX-2 mRNA induction was observed after 9-12 hr. Thus, my data suggest that at least certain of the *cis*-elements and their cognate transcription factors participate at different times and to different degrees to regulate the prolonged COX-2 gene expression that occurs during the development of an inflammatory response.

For mom and dad

for their constant support, guidance, and abundant love.

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PREFACE

This thesis is composed of three related manuscripts, each representing a chapter (1-3). These chapters either have been or will be submitted to peer-reviewed journals. The introduction (chapter 1) summarizes background information, the explosive progress and unresolved questions in the field. This chapter will be submitted as a review article to Biochemica et Biophysica Acta, Molecular and Cell Biology of Lipids with minor modifications. Chapter 2 consists of studies on the dynamics and mechanisms of sustained COX-2 transcriptional regulation over 12 hr period of LPS-stimulated murine macrophages. This chapter contains the manuscript that has been submitted to Journal of Immunology with some modifications in the result section. The third chapter forms the core of a manuscript in preparation.

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LIST OF ABREVIATIONS

AP-1	Activator Protein-1
ATF	Activating Transcrtiption Factor
ASK1	Apoptosis Signal-regulating Kinase
bFGF	basic Fibroblast Growth Factor
CAK	Ceramide Activated Kinase
C/EBP	CAAT Enhancer Binding Protein
cAMP	Cyclic Adenosine Monophosphate
COX	Cyclooxygenase
CR	Cytokine Receptor
CRE	cAMP Response Element
CREB	cAMP Response Element Binding Protein
DMEM	Dulbecco's Modified Eagle's Medium
ECSIT	Evolutionary Conserved Signaling Intermediate in Toll
EGF	Epidermal Growth Factor
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular signal-Regulated Kinase
GF	Growth Factor
GFR	Growth Factor Receptor
HFF	Human Foreskin Fibroblast
I-κb	Inhibitor of κB
IKK	Inhibitor of κB Kinase
IL-1	Interleukin-1
IL-1R	Interleukin-1 Receptor
IRAK	Interleukin-1 Receptor Associated Kinase
IRF	IFN (Interferon) regulatory factor
JNK	c-Jun N-term Kinase
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MAPKAP-K1	MAPK- Activated Protein Kinase-1
MBD	Membrane Binding Domain
MEK	See MKK
MEKK	See MKKK
MKK	Mitogen Activated Protein Kinase Kinase, also called MEK
MKKK	Mitogen Activated Protein Kinase Kinase Kinase, also called MEKK
MSK-1, -2	Mitogen and stress Activated Kinase-1, -2
MyD88	Myeloid Differentiation Factor
NF-κB	Nuclear Factor-kappa B
NIK	Nuclear Factor-kappa B Inducing Kinase
p50	Rel protein 50, NF-κB transcription factor
p65	Rel protein 65, NF-κB transcription factor
PAK	p21 Associated Kinase
PDGF	Platelet Derived Growth Factor

PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGHS	Prostaglandin H Synthase, Prostaglandin Endoperoxide Synthase
PGI_2	Prostaglandin I_2
PI3K	Phosphatidyl-Inositol-3-Kinase
PKA	cAMP dependent Protein Kinase
PKC	Calcium dependent Protein Kinase
PLC	Phospholipase C
PMA	Phorbol 12-Myristate 13-Acetate
PTH	Parathyroid Hormone
RIP	TNF Receptor Interacting Protein
SP	Signal Peptide
SRE	Sterol Response Element
TAE	Tris-Acetate EDTA
TAK-1	Transforming growth factor-beta Activated Kinase-1
TBK-1	TANK-Binding Kinase-1
TCF	T-Cell Factor
TGF-α -β	Transforming Growth Factor-α -β
TIR	Toll/IL-1 Receptor
TKR	Tyrosine Kinase Receptor
TLR4	Toll-Like Receptor-4
TNF-α -β	Tumor Necrosis Factor-α -β
TNFR	Tumor Necrosis Factor Receptor
TPA	Phorbol 12-Tetradecanoate 13-Acetate
TRADD	Tumor Necrosis Factor Receptor Associated Death Domain
TRAF	Tumor Necrosis Factor Receptor Associated Factor
TRAM	TRIF-related Adaptor Molecule
TRIF	TIR domain-containing adaptor-inducing IFN-β

CHAPTER 1

LITERATURE REVIEW
CYCLOOXYGENASE GENE EXPRESSION AND PROTEIN DEGRADATION

Summary

Cyclooxygenase-1 (COX-1) expression is induced during development, and COX-1 mRNA and COX-1 protein are very stable. This combination of properties can explain why COX-1 protein levels are relatively high and remain constant in most cells expressing this enzyme. COX-2 is usually expressed inducibly in association with cell replication or differentiation and both COX-2 mRNA and COX-2 protein have short-lives relative to those of COX-1. Consequently, COX-2 protein is typically present for only a few hr after its synthesis.

There are major gaps in our understanding of (a) the mechanisms involved in the induction of COX-1 gene expression; (b) the way in which COX-2 gene expression is regulated and (c) the molecular basis for the rapid turnover of COX-2 protein. Here we review what is currently known about these topics. In particular, we develop the two concepts (a) that COX-2 gene expression can be regulated by numerous signaling pathways that generate trans-acting factors that, in turn, interact interactive with different combinations of *cis*-elements in a coordinate manner and (b) that the relative contribution of each *cis*-element depends on the cell type, the stimulus and the time following the stimulus.

Introduction

Cyclooxygenase (COX, PGHS) metabolizes arachidonic acid, hydrolyzed from cell membrane phospholipids by phospholipase A_2, to prostaglandin endoperoxide H_2 (PGH_2), the precursor of thromboxane A_2 (TxA_2) and prostaglandins (PGs) (Fig. 1). Prostaglandins are lipid mediators that normally act in a paracrine and autocrine manner to coordinate intercellular events stimulated by a circulating hormone. TxA_2 from platelets is an important mediator of platelet aggregation. Prostaglandins play critical roles in normal physiological process such as stomach mucus secretion, kidney water excretion, ovulation, fertilization, fetal development, and parturition. Moreover, prostaglandins are involved in the pathophysiology of tumorigenesis, inflammation, fever and pain transmission (Fig. 2) (1, 2).

Nonsteroidal anti-inflammatory drugs (NSAIDs), which act primarily via inhibition of cyclooxygenase activity and abrogation of prostaglandin biosynthesis, are commonly used for the treatment of acute inflammation, pain, fever, and chronic inflammatory diseases such as asthma, rheumatoid arthritis, and inflammatory bowel disease (IBD). NSAIDs are also used for the prevention of coronary artery thrombosis, Alzheimer's diseases, and gastrointestinal and breast cancer (3).

There are two unique, yet highly related COX isozymes--a housekeeping COX-1 and an inducible COX-2. Although COX-1 is constitutively expressed in most cells and tissues, it is developmentally controlled and can be upregulated by tumor-promoting phorbol ester and growth factors as seen with primary megakaryocytes and megakaryoblast cell lines (Table I) (4-6). In contrast, COX-2 expression is highly regulated and can rapidly induced by bacterial endotoxin (LPS), cytokines such as IL-1, IL-2, and TNF-α, growth factors, and the tumor promoter phorbol myrisate acetate

(PMA) depending on the cell type (7). Brain, kidney, pancreatic β-cells, and colon carcinomas exhibit constitutive COX-2 expression references for all but this topic is not addressed in this review.

The ability of the COX enzymes to orchestrate the complex physiologic functions that are mediated by prostaglandins reflects an elaborate interplay between the two isoforms. The differences in these isozymes in structure, expression level, and regulation could contribute to the unique roles of each enzyme. COX-1 and COX-2 have approximately 60% amino acid sequence identity and their active site structures are highly conserved. Both are homodimeric proteins with a molecular mass of roughly 71 KDa. Their active sites both consist of a hydrophobic channel, and the amino acids in this region are almost identical; however, COX-2 has a larger and more accessible side pocket than COX-1 (8, 9). COX-1 and COX-2 proteins have some sequence and structural differences. These differences include different signal peptides and significant sequence differences in their membrane binding domains (10). Most notably, COX-2 but not COX-1 contains a unique 18 amino acid cassette near the C-terminus (Fig. 3).

While little is known about the transcriptional regulation of COX-1 gene and the mechanism of COX-1 enzyme degradation, the regulation of COX-2 has been investigated extensively. Numerous studies indicate that COX-2 expression is regulated at the transcriptional and post-transcriptional levels. In most settings COX-2 expression is mainly regulated at the level of gene transcription although in a few cases COX-2 induction is primarily due to mRNA stabilization. In this review, we focus on recent advances in our understanding of the transcriptional regulation of COX isozymes and their degradation.

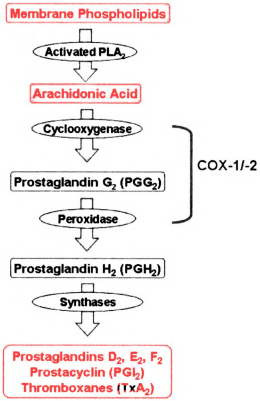
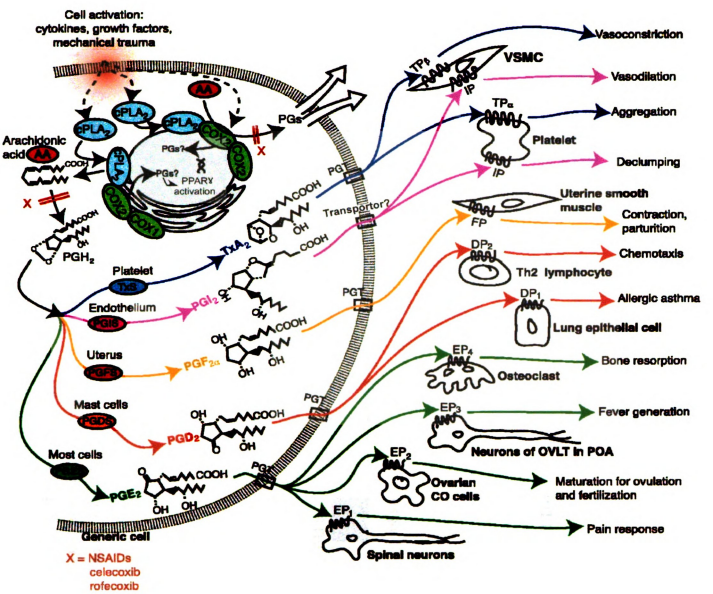


Figure 1. Arachidonic acid (AA) metabolism.

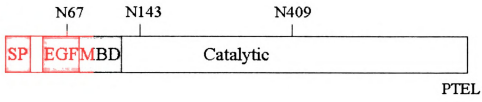
Prostaglandin biosynthesis is mediated by either the COX-1 or the COX-2 enzyme. Phospholipase A_2, which is activated by a number of mechanical or hormonal factors, releases AA from cell membrane phospholipids. Free AA is then bound by COX, which has cyclooxygenase and peroxidase activity, converted into unstable prostaglandin G_2 (PGG_2) and then prostaglandin H_2 (PGH_2). PGH_2 is transformed into prostaglandin, prostacyclin, and thromboxane by in a cell specific manner by PGE, prostacyclin and thromboxane synthases.

Figure 2. Prostaglandin synthesis and actions.

When a cell is activated by various stimuli, arachidonic acid (AA) is released from membrane lipids by cytosolic phospholipase ($cPLA_2$) and metabolized by COX-1 or COX-2, resulting in the production of PGH_2. There is also *de novo* COX-2 enzyme synthesis induced by a host of factors (top). PGH_2 can be converted into different prostacyclins (PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2) and thromboxane A_2 (TxA_2). These prostaglandins are transported from the cell through the prostaglandin transporter (PGT) to exert autocrine or paracrine actions via perhaps a family of prostaglandin receptors including EP_1, EP_2, EP_3, EP_4, DP_1, DP_2, FP, IP, TP_α, and TP_β associated with the cell types indicated above. Figure adapted from Funk *et. al.* (11)


Cell activation:
cytokines, growth factors,
mechanical trauma
cPLA2
AA
Arachidonic acid
COX-1
COX-2
PGs
PGs?
PPARγ activation
X
PGH2
TXA2
Platelet
TXS
PGI2
Endothelium
PGIS
Uterus
PGFS
PGF2α
Mast cells
PGDS
PGD2
Most cells
PGES
PGE2
Generic cell
X = NSAIDs
celecoxib
rofecoxib
Transporter?
PGT
TPs
VSMC
IP
Vasoconstriction
Vasodilation
TPα
Platelet
Aggregation
Declumping
Uterine smooth muscle
FP
Contraction, parturition
DP2
Chemotaxis
Th2 lymphocyte
DP1
Allergic asthma
Lung epithelial cell
EP4
Bone resorption
Osteoclast
EP3
Fever generation
Neurons of OVLT in POA
EP2
Ovarian CO cells
Maturation for ovulation and fertilization
EP1
Pain response
Spinal neurons

COX-1/PGHS-1



COX-2/PGHS-2

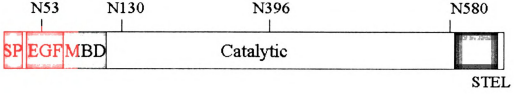
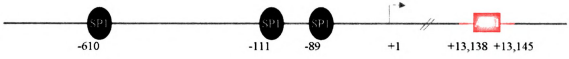


Figure 3. Comparison of the protein structures of the cyclooxygenase isoforms COX-1 and COX-2. Both isoforms have a signal peptide (SP), an epidermal growth factor (EGF)-like domain, a membrane-binding domain (MBD), and a catalytic domain. The C-terminal PTEL and STEL sequences in COX-1 and COX-2, respectively, represent an ER targeting signal. The glycosylation sites are also shown; note the additional glycosylation site in the COX-2 protein. COX-2 has a shorter signal peptide than COX-1, and there is a unique 18 amino acid insertion near the C-terminus of COX-2 (residues 595-612).

1. The regulation of COX-1 gene expression

The human COX-1 gene, located on chromosome 9, is approximately 22 kb in length and contains 11 exons (12, 13). The COX-1 promoter lacks a TATA or CAAT box, has a high GC content, and contains several transcriptional start sites. All of these properties are characteristic of "housekeeping" genes (14, 15). Although COX-1 protein is constitutively expressed in most tissues, COX-1 is upregulated by PMA in some cell types including monocytes (16), human embilical vein endothelial cells (HUVEC) (17), and primary megakaryocytes and megakaryoblasts (5, 6) as they differentiate during development (Table I). COX-1 is also induced by shear stress in HUVEC cells (18), by bradykinin in the gallbladder (19), and by estrogen in endothelial cells (20). Within the 5' flanking region of the human COX-1 promoter there are three functional Sp1 binding sites at -610, -111, and -89 relative to the ATG start site (Fig. 4). In HUVECs the Sp1 motifs at -610 and -111 contribute to constitutive expression but not to PMA-induced expression of the COX-1 gene (17). The Sp1 binding site at -111 is required for PMA-induced COX-1 transcription in the megakaryoblast cell line MEG-01 (21) and for estradiol induction of COX-1 in ovine endothelial cells (20) where both the Sp1 site at -111 and to an even greater extent the Sp1 site at -89 are required for COX-1 induction. These three Sp1 sites are the only functional regulatory elements that have been described within 2 kb upstream of the ATG start codon. However, there is a highly conserved AP-1 site in intron 8 of the COX-1 gene that interacts with the -111 SP-1 site of the promoter to regulate PMA-induced expression of COX-1 in MEG-01 cells (21).

Human COX-1 Promoter



Human COX-2 Promoter

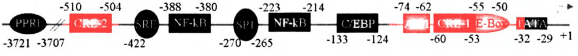


Figure 4. Schematic representation of the functional regulatory elements in the human COX-1 and COX-2 genes. The *cis*-acting elements are noted in the shaded boxes and their location relative to the transcriptional start site are noted above or below each element. Note that the COX-1 promoter lacks a TATA box. Abbreviations: CRE, cAMP Response Element; SRE, Sterol Response Element; NF-κB, Nuclear Factor-kappa B; C/EBP, CAAT Enhancer Binding Protein; AP-1, Activator Protein-1

2. The regulation of COX-2 gene expression

The human COX-2 gene, located on chromosome 1, is about 8.3 kb long and has 10 exons. Except for the first exon the intron/exon boundaries of the COX-1 and COX-2 genes are the same (14, 22). There are two major transcripts of COX-2--a 4.5 kb full length mRNA and a 2.6 kb polyadenylated variant that lacks the terminal 1.9 kb of the 3'-untranslated region (UTR). The 3'-UTR of the COX-2 gene contains 23 copies of the 'ATTTA' RNA instability element that participates in post-transcriptional regulation of COX-2 expression (23). Sequence analysis of the 5'-flanking region of the human COX-2 gene has identified several potential transcriptional regulatory elements, including a peroxisome proliferator response element (PPRE), two cyclic AMP response elements (CRE), a sterol response element (SRE), two nuclear factor kappa B (NF-κB) sites, an SP1 site, a CAAT enhancer binding protein (C/EBP, or nuclear factor for interleukin-6 expression (NF-IL6)) motif, two AP-2 sites, an E-box, and a TATA box (Fig. 4). The promoter regions of COX-2 genes have the sequences of typical immediate early genes (14). There are some subtle interspecies differences in the sequences of the human, mouse, rat, cow and horse COX-2 genes. For example, the mouse COX-2 promoter has one NF-κB motif and two C/EBP sites instead of the two NF-κB sites and one C/EBP motif found in the human COX-2 promoter. Transcriptional regulation of the COX-2 gene is very complex in that it can involve numerous signaling pathways, and the mechanism varies depending on the specific stimulus and the cell type. Here, we compare COX-2 gene regulation in several different cell types (Table II) and the regulatory elements (fig. 4) and signaling pathways (fig. 5) involved in each system.

Table 1. COX-1 inducers

Cell type	Inducers
Fibroblasts	IL-1β (24), TGFβ (25)
Vascular endothelial cells	VEGF (26), Shear stress (18), Estrogen (Estradiol-17β) (20)
Tracheal epithelial cells	Phorbol myristate acetate (PMA) (27)
Gallbladder	Bradykinin (BK) (19)
Neuroblastoma	Retinoic acid (28)
Monocytes/Megacaryoblasts	TPA (16, 29), PMA (5, 6, 16), Tobacco carcinogens (30)

Table 2. COX-2 inducers

Cell type	Inducers	Signaling pathways
Fibroblasts		
NIH 3T3 fibroblasts	Serum, PDGF, PMA, v-src (31-33)	Ras/MEKK-1/JNKK(MKK4)/JNK (31-33)
	IL-1β, TNFα, PGE_2, PGE_3 (34)	Ras/Raf-1/MAPKK/ERK-1/2 (31-34)
Human foreskin fibroblasts (HFF)	PMA (35), TNFα, IL-1β, LPS (36)	C/EBP (35), AP-1 (35), NF-κB (37, 38) (36)
Human intestinal myofibroblasts	IL-1α (39)	PKC, NF-κB, ERK-1/2, JNK, p38 (39)
Human synovial fibroblasts	IL-1β (40)	p38, NF-κB, AP-1/ATF (40)
Endothelial cells		
Bovine arterial endothelial cells (BAECs)	LPS, PMA (41)	NF-κB, C/EBP (41)
Human umbilical vein endothelial cells	hypoxia (42, 43), VGEF (43)	NF-κB, Sp1 (42), PKC (43)
(HUVEC)	PMA, IL-1β (43), TNF-α (43), LPS (44),	
	thrombin, cholesterol deprivation (45),	

	IL-1, vanadate (46), platelet derived thromboxanse A_2 (47)	
Smooth Muscle cells		
Human pulmonary artery smooth muscle cells (HPASMC)	BK (48), TGFβ, IL-1β, hypoxia (49)	G_s
Human airway smooth muscle cells (HASM)	BK, IL-1β (50), indomethacin, flurbiprofen, NS-398, 15d-PGJ_2 (51)	NF-κB, CREB, C/EBP (50) PPARγ (51)
Epithelial cells		
Human mammary epithelial cells (HER)	PMA (52)	PKC, ERK-1/2, p38, JNK (52)
Human gastric epithelial cells (hGECs)	PMA, *Helicobacter pylori* (53)	MEK-1/2, CREB, USF-1/2 (53)
Granulosa Cells	Gonadotropin, forskolin, FSH, phorbol didecanoate, LH (54-58)	PKA (54-58)
Bone		
MC3T3-E1 osteoblasts	Bone morphogenic proteins (BMP-2) (59), bFGF, EGF, TGF-α/β, IL-1, (60), thrombin, shear stress (61), TNF-α (62), parathyroid hormone (PTH), PMA (63), BK, epinephrine, prostaglandins, serum (60, 63)	MEKK, JNK, NF-κB (60) C/EBP, AP-1, CREB (61) C/EBP, NF-κB (62),
Monocytes/Macrophages		

Human U937 monocytic cells	PMA, LPS, TNF-α, IL-1 (64), MP (65)	C/EBP, NF-κB (64) PI3K/PKC, ERK-1/2, p42/p44 MAPK p38, JNK-1 (65)
THP-1 monocytic cells	High glucose (HG) (66, 67)	PKC, p38 MAPK, CREB, NF-κB (66, 67)
RAW 264.7 macrophages	Catalase (68), LPS, IL-1, TNFα, peptidoglycan (69), double-stranded RNA (70),	NF-κB, PI3K, ERK, p38, JNK (68) NF-κB, Ras/Raf-1, ERK (69) NF-κB (70)

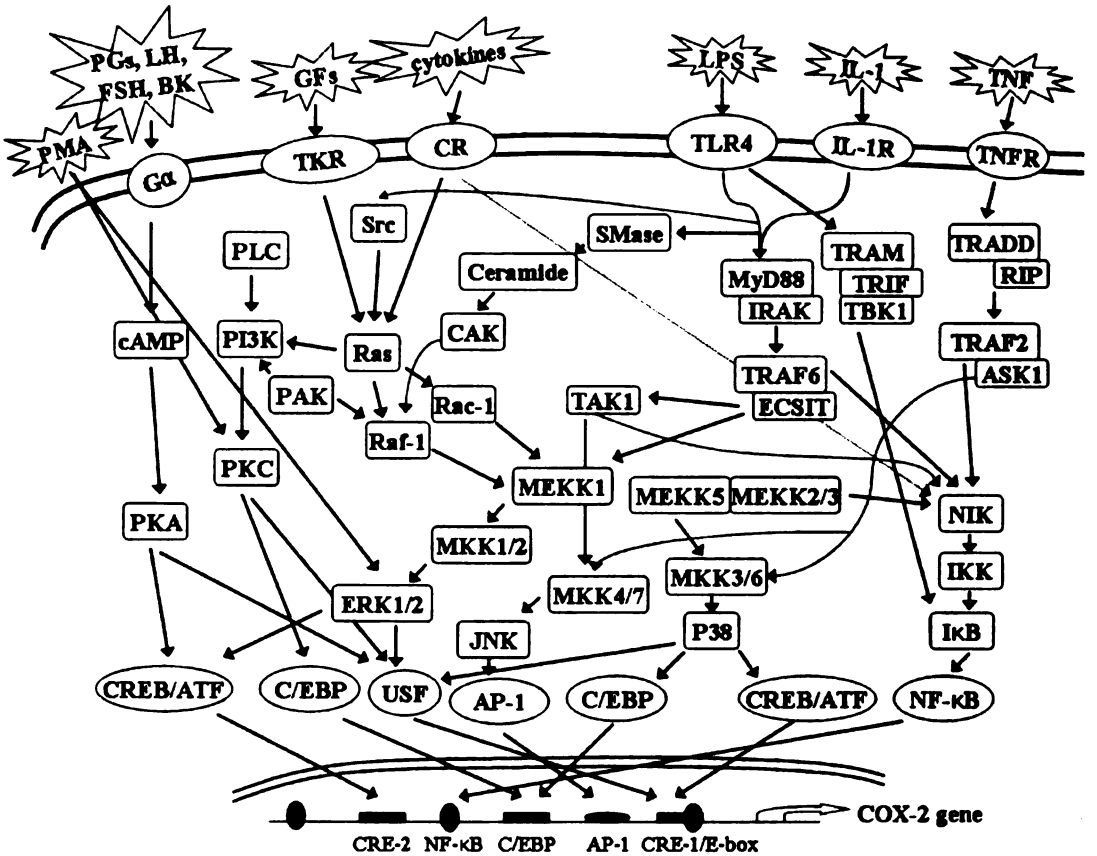


Figure 5. Schematic representation of COX-2 gene induction signaling pathways.

2.1. Transcriptional Regulation of COX-2 in Fibroblasts

COX-2 expression is upregulated in murine NIH 3T3 fibroblast lines in response to fetal calf serum, PDGF, PMA, v-src (31, 32, 71), IL-1β, TNFα, PGE_2, and PGE_3 (Table II) (34). v-Src oncogene-induced COX-2 expression in NIH 3T3 cells is mediated mainly by CREB and AP-1 transcription factors working through CRE-1. AP-1 activity at the CRE-1 is modulated by the Ras/MEKK-1/JNKK(MKK4)/JNK pathway leading to c-Jun phosphorylation and the Ras/Raf-1/MAPKK/ERK1/2 pathway that activates secondary response genes such as c-fos that form heterodimers with c-Jun (31, 71). In addition to the CRE-1 site, there is an E-box element within the mCOX-2 promoter that is also required for v-src-induced COX-2 expression in NIH 3T3 fibroblasts. Serum, PGE_2 and PGE_3 also induce COX-2 mRNA through Ras/JNK- and Ras/ERK-mediated signaling pathways (34). Transcriptional activation of COX-2 by serum, PGE_2, and PGE_3 requires a C/EBP site and the CRE-1 site; however, the NF-κB site and E-box element are not involved. This indicates that COX-2 gene activation is moderated by distinct combinations of *cis*-regulatory elements and trans-acting factors that are specific for different stimuli.

Human foreskin fibroblasts (HFF) express COX-2 protein after 2 hrs of PMA treatment and the protein level increases over a 4 hr period (35). In contrast, serum-treated NIH 3T3 fibroblasts exhibit more transient COX-2 expression lasting just 2 hrs. COX-2 transcriptional activation in PMA-treated HFF is through the CRE-1 and the C/EBP element. In the basal state, C/EBPδ binds to both the C/EBP site and the CRE-1. In response to PMA, C/EBPδ at the C/EBP site is replaced by C/EBPβ, C/EBPβ-LAP (activating form) or C/EBPβ-LIP (inhibitory form); binding of C/EBPδ to the CRE-1 site is unchanged even after 4 hr of stimulation. PMA stimulation induces COX-2 gene

expression in HFF cells by reducing C/EBPδ protein levels and increasing C/EBPβ phosphorylation which enhances its DNA binding affinity and increases C/EBPβ-LAP binding to the C/EBP element. These changes lead to recruitment of the coactivator p300 to the transcriptional machinery to initiate transcription.

PMA treatment of HFF also induces the binding of the c-Jun/c-fos heterodimer to the CRE-1 site. Stimulation by TNFα relies upon the inducible binding of the p65/p50 heterodimer to the NF-kB site (36-38). The CRE-1 motif is also bound by CREB2/ATF2 transcription factors; however, their recruitment to the COX-2 promoter does not depend upon PMA or TNFα stimulation. In addition to PMA and TNFα, IL-1β and LPS can induce COX-2 expression in HFF.

p300 is the predominant transcriptional coactivator in HFF with CBP being barely detectable (36). p300 and PCAF (p300/CBP associated factor) are recruited by transcription factors that are bound to their cognate *cis*-elements within the COX-2 promoter. p300 and PCAF direct gene expression by acetylating nearby histone tails leading to a "loosened" chromatin structure that is more accessible to transactivators. HDAC-1 (histone deacetylase-1), on the other hand, cleaves acetyl moieties from histone tails and thus negatively regulates COX-2 expression. A balance between acetylation and deacetylation is required to maintain COX-2 expression at physiologically relevant levels.

IL-1α induces COX-2 expression in human intestinal myofibroblasts (39). Activation of protein kinase C (PKC), NF-κB, ERK-1/2, and JNK are all required for optimal COX-2 transcriptional activation whereas p38 activation is involved in the stabilization of COX-2 mRNA. COX-2 mRNA levels increase after 1 hr of IL-1α treatment, peak after 8 hr and then remain the same for the next 16 hr. COX-2 protein

expression is observed after 4 hr of IL-1α stimulation and is maximal at 16-24 hr. In contrast, with serum induction, COX-2 mRNA levels decrease to baseline after 4 hr.

In human synovial fibroblasts, IL-1β treatment also results in prolonged expression of COX-2 which is mediated by mRNA stabilization involving p38 MAP kinase (40). IL-1α stimulation induces binding of p65/p50 to the NF-κB motif and of AP-1/ATF transcription factors to the CRE-1 while binding of nuclear proteins to the C/EBP site is not inducible.

2.2. Transcriptional Regulation of COX-2 in Endothelial Cells

LPS and PMA act synergistically to induce COX-2 mRNA in bovine arterial endothelial cells (BAECs) (72). This COX-2 transcriptional activation is mediated by the NF-κB and C/EBP elements and unlike in fibroblasts, the CRE-1 motif appears not to be required. However, the CRE-1 element is required for COX-2 induction when C/EBPδ is activated independently (i.e. by transfection), suggesting that there is an interplay between the CREB and C/EBPδ transcription factors bound to their cognate sites during COX-2 gene activation.

COX-2 expression and prostaglandin synthesis in human umbilical vein endothelial cells (HUVEC) can be stimulated by physical stimuli and by agents, such as, PMA, IL-1β, TNF-α, LPS, vascular endothelial growth factor (VGEF), thrombin, hypoxia, vanadate (an inhibitor of protein-tyrosine phosphatases), cholesterol deprivation and platelet-derived thromboxane A_2 (Table II) (42-47). Hypoxia causes increased binding of p65 to the proximal NF-κB site of the COX-2 promoter in HUVEC without influencing the levels of cytoplasmic p65 or IκBα, an inhibitory protein that binds cytoplasmic p65/p50. Promoter deletion analysis implicate other regulatory *cis*-

elements and their cognate transcription factors such as C/EBP, AP-1, and the CRE-1/E-box in this response, however their roles in this system have yet to be determined. Hypoxic HUVEC also exhibit nuclear localization of Sp1 while Sp3 protein levels are unaffected, thus elevating the Sp1/Sp3 ratio in the nucleus. Sp1 and Sp3 protein bind to the SP1 motif in the COX-2 promoter and mediate COX-2 transcription (42). High concentrations of cholesterol down regulate COX-2, whereas cholesterol deprivation upregulates COX-2 gene expression in endothelial cells. Cholesterol-dependent COX-2 regulation is mediated by sterol response element binding protein (SREBP) through an SRE located at -422 from the transcription start site (45).

2.3. Transcriptional Regulation of COX-2 in Smooth Muscle Cells

Bradykinin (BK) binds to specific cell surface G protein-coupled receptors and induces COX-2 expression in human pulmonary artery smooth muscle cells (HPASMC) (48). COX-2 mRNA increases 2 fold after 1 hr of BK treatment and returns almost to basal levels within 4 hrs indicating that COX-2 gene activation is transient in this system. BK-induced COX-2 expression is transcriptionally regulated by CREB binding to the CRE-1 motif. COX-2 transcription is also activated by cytosolic phospholipase A_2 ($cPLA_2$)-mediated arachidonic acid release and an autocrine loop involving the action of endogenous PGE_2 on G_s-linked EP2 and EP4 receptors (48, 50). BK activates IL-1β mRNA expression in human lung fibroblasts by activating NF-κB, and in HeLa cells BK activates C/EBP during IL-8 gene expression. However, COX-2 gene activation by BK in HPASMC does not involve either the NF-κB or C/EBP motifs. Thus, BK can induce transcription in a gene- and transcription factor-specific manner.

BK and IL-1β activate COX-2 expression in human airway smooth muscle cells

(HASM) in a stimulus-specific manner (50). BK induces COX-2 protein expression more quickly (1 hr) than does IL-1β (2 hr). BK induction is also more transient – COX-2 protein disappears within 16 to 24 hrs. With IL-1β-stimulated COX-2 protein can be sustained beyond 24 hrs. Both stimuli involve CREB, but not c-Jun binding to the CRE-1 element. However, IL-1β induced COX-2 transcription requires two other *cis*-elements, the NF-κB and the C/EBP sites, for maximal COX-2 gene expression. COX-2 transcriptional regulation by BK and IL-1β involving different sites in the COX-2 promoter may due to the different chromatin structure resulting from different patterns of histone modification (50, 73).

While NSAIDs inhibit COX activity, there has been speculation that they induce COX-2 expression in HASM (51). Indomethacin, flurbiprofen, NS-398 (a selective COX-2 inhibitor) and 15d-PGJ_2 induce COX-2 expression and enhance IL-1β-induced COX-2 expression through the peroxisome proliferator response element (PPRE) in the COX-2 promoter. NSAID treatment causes nuclear translocation of PPARγ but not NF-κB in HASM cells. PPARγ binds to the PPRE of the COX-2 promoter and can transactivate the gene.

2.4. Transcriptional Regulation of COX-2 in Epithelial Cells

In contrast to what is reported for NSAID-treated smooth muscle cells, PPARγ ligands such as ciglitazone and 15d-PGJ_2 inhibit PMA-mediated induction of COX-2 in human mammary epithelial cells (184B5/HER) (52). PMA-stimulated COX-2 induction in these cells is mediated by the CRE-1. PMA treatment leads to c-Jun, c-Fos, and ATF-2 binding to the CRE-1 site; this can be prevented by PPARγ ligands by inhibition of AP-1 activity. PMA treatment activates PKC, ERK-1/2, p38, and JNK signaling pathways

in human mammary epithelial cells. Activated ERK-1/2 induces c-fos expression, p38 phosphorylates ATF-2 and ATF-2 dimerizes with c-Jun and induces more c-Jun expression. Activated JNK both induces c-Jun expression and phosphorylates c-Jun, allowing it to activate the COX-2 gene expression. PMA-induced COX-2 transcription in this system also requires a functional CBP/p300 coactivator complex having HAT activity. Retinoic acid (RA) and other nuclear receptor ligands, and carnosol, a phenolic antioxidant isolated from rosemary oil, suppress PMA-mediated COX-2 transcription. RA is thought to downregulate COX-2 expression by a receptor-dependent mechanism whereby the ligand-bound receptor complex sequesters the CBP/p300 that is available for AP-1 mediated induction of COX-2. Carnosol reduces binding of AP-1 to the CRE-1 element by inhibiting PKC, ERK1/2, p38, and JNK signal transduction pathway (74).

Helicobacter pylori and PMA stimulate transient COX-2 transcription (1.5 hr to 4.5 hr after stimulation) in human gastric epithelial cells (hGECs) through the CRE-1/E-box, but not via the C/EBP, AP-1 or NF-κB sites (53). *Helicobacter pylori* activates the MEK-1/2 kinase cascade, which in turn activates CREB and USF-1/2 transcription factors. Unlike in PMA-stimulated HER cells, in hGECs CREB protein but not c-Jun binds to the CRE-1 site. USF-1/2 binds to the E-box and activates COX-2 gene transcription. *Helicobacter pylori* treatment does not require any *cis*-regulatory element other than the CRE-1/E-box element. Whether there are interactions between CREB and USF-1/2 transcription factors bound to this site remains to be determined.

2.5. Transcriptional Regulation of COX-2 in Granulosa Cells

Prostaglandins play important roles in several aspects of female reproduction. For example, follicular prostaglandin levels increase dramatically prior to ovulation (75)

and NSAIDs such as indomethacin block ovulation (75). COX-2 deficient female mice are infertile because of problems with ovulation, fertilization, implantation and decidualization (76).

COX-2 is induced by gonadotropins in granulosa cells prior to ovulation, and also by forskolin, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and phorbol didecanoate all acting through the protein kinase A (PKA) signaling pathway (54-58). The duration of COX-2 induction in granulosa cells varies from 2-4 hr in rats to 18-30 hr in cows and mares. Bovine and rat COX-2 promoter analyses revealed that the proximal 150-200 bp upstream of the transcriptional start site is sufficient to confer inducible promoter activity. This region of the COX-2 promoter contains the C/EBP, CRE-1 and E-box elements, and mutational analyses have suggested that only the E-box element is required for promoter activity in rat granulosa cells but that both the C/EBP and E-box elements are required in bovine granulosa cells. Supershift EMSAs have established that USF-1 and USF-2 bind to the E-box element of both the bovine and the rat COX-2 promoters and that gonadotropin treatment has no effect on the binding of protein complexes to the E-box. In contrast, C/EBPβ binds to the bovine C/EBP site in the absence of stimulation and gonadotropin treatment decreases C/EBPβ binding. However, in rat granulosa cells, there is no C/EBPβ binding to the C/EBP site prior to treatment but C/EBPβ binding rapidly increases upon treatment with gonadotropin (57, 58). This difference may explain the molecular basis for the variation in the duration of COX-2 expression among species.

Recent studies have indicated that phosphorylation of USF is involved in the regulation of COX-2 promoter activity in granulosa cells (77). PKA-mediated USF phosphorylation increases USF binding to the E-box promoter element thereby enhancing

COX-2 promoter activity. In contrast, activation of the PKC pathway with PMA had no stimulatory effect on the COX-2 promoter. Thus, activation of the cAMP/PKA pathway by luteinizing hormone and consequent phosphorylation of USF proteins that bind to the E-box appears to be essential for induction of COX-2 in granulosa cells.

2.6. Transcriptional Regulation of COX-2 in Bone

COX-2 plays a significant role in bone resorption and formation. The regulation of COX-2 expression has been extensively studied in MC3T3-E1 osteoblasts. MC3T3-E1 cells established from newborn mouse calvaria, have the capacity to differentiate into osteoblasts and osteocytes and to form calcified bone tissue *in vitro*. COX-2 can be up-regulated by many stimuli in MC3T3-E1 cells including BMP-2, bFGF, EGF, TGF-α/β, IL-1, TNF-α, PTH, thrombin, bradykinin, forskolin, epinephrine, and prostaglandins (PGI_2, PGE_2, $PGF_2\alpha$) or their stable analogues (Table II) (14, 59-63).

TNF-α treated MC3T3-E1 cells exhibit a triphasic change in COX-2 mRNA expression. There is an initial increase that reaches a maximum 2 hr after TNF-α stimulation. This is followed by a decrease at 3 hr, and then a second increase in COX-2 mRNA at 6-12 hr. The second increase is due to PGE_2 produced by the COX-2 formed in the first phase (62). Binding of p65/p50 to the NF-κB site and C/EBPβ to the C/EBP-1 site at 1 hr are necessary for TNF-α induced COX-2 expression in MC3T3-E1 osteoblasts. However, different transcription factors and *cis*-elements may be involved during the first and second increases in COX-2 transcription.

bFGF, PDGF, PGE_2 or a combination of TNF-α and IL-1β stimulation causes after 4 hr the activation of c-Jun, MEKK and JNK signaling pathways in MC3T3-E1 (60). Promoter analysis identified a second C/EBP motif in the murine COX-2 promoter that is

required together with the C/EBP-1 site for optimal promoter activity. C/EBPβ and C/EBPδ transactivate murine COX-2 gene expression via C/EBP sites. The CRE-1 element is necessary for COX-2 transcriptional activity while neither the NF-κB site as the E-box are not required for bFGF, PDGF, PGE_2, or TNFα+IL-1β induced COX-2 expression in MC3T3-E1 osteoblasts.

When MC3T3-E1 cells are treated with PMA or serum for 3 hr, the -371/+70 region of the COX-2 promoter, which does not include the CRE-2 or the NF-κB sites, is sufficient to activate COX-2 transcription (63). PMA or serum treatments cause inducible binding of c-Jun and c-fos to the AP-1 site and constitutive binding of nuclear protein to the CRE-1 site. This suggests that there is a cooperative interaction between transcription factors bound to the AP-1 site and the CRE-1 in *trans*-activating COX-2 transcription. The CRE-1 site is quantitatively more important for the serum response and the AP-1 site is more important for the PMA response (63).

Mechanical loading is crucial for maintaining bone mass and integrity. This generates extracellular matrix deformation and fluid flow. Mechanical stimuli such as loading and fluid shear stress cause the production of signaling factors. In bone forming cells, prostaglandins are formed that modulate the overall process of bone metabolism. Fluid sheer stress induces COX-2 expression through a process involving the activation of a cytoskeleton-associated Ca^{2+} channel, phospholipase C, PKA, PKC and phospholipase A2 (78, 79). COX-2 mRNA levels increase after 1 hr of fluid sheer stress and show a sustained increase for up to 9 hr of treatment. Sustained COX-2 gene activation likely occurs via the PKA pathway. The CRE-2, NF-κB, C/EBP-2 and E-box appear not play roles in fluid sheer stress-mediated COX-2 transcription in MC3T3-E1 cells. Instead, the process involves the C/EBP-1 site bound inducibly by C/EBPβ, the

AP-1 element bound inducibly by c-Jun/AP-1 transcription factors, and the CRE-1 constitutively bound by CREB. This is similar to the mechanisms by which PMA and serum induce COX-2 gene activation.

2.7. Transcriptional Regulation of COX-2 in Monocytes/Macrophages

Monocytes/macrophages are crucial to the development of the immune response because of their ability to present antigens and secrete mediators of inflammation including cytokines and prostaglandins. These products regulate cell proliferation, differentiation, and, in general, the acquisition of immune functions. Regulation of prostaglandin synthesis in monocytes is important in immune responses

Inflammation is a nonspecific response of the immune system to damaged cells and is characterized by redness, pain, heat and swelling of tissue (80). Although there are many components to inflammation, prostaglandin E_2 (PGE_2) is known to be highly associated with inflammation which is substantially reduced by NSAIDs. PGE_2 acts as vasodilator allowing more blood to flow through affected tissues, and the increased blood flow generates the heat and redness of inflammation. Prostaglandins play a direct role in sensing pain, and also cause the emigration of phagocytes through capillary walls.

LPS, also known as lipopolysaccharide or endotoxin, is an outer membrane constituent of Gram-negative bacteria such as *E.coli* and a potent activator of innate immune responses that result in the production of pro- and anti-inflammatory mediators. The LPS molecule that constitutes the outer layer of the outer membrane is composed of a hydrophobic region, called Lipid A, which is attached to a hydrophilic linear polysaccharide region, consisting of the core polysaccharide and the O-specific polysaccharide (Fig. 6A). The Lipid A head of the molecule is the toxic component and

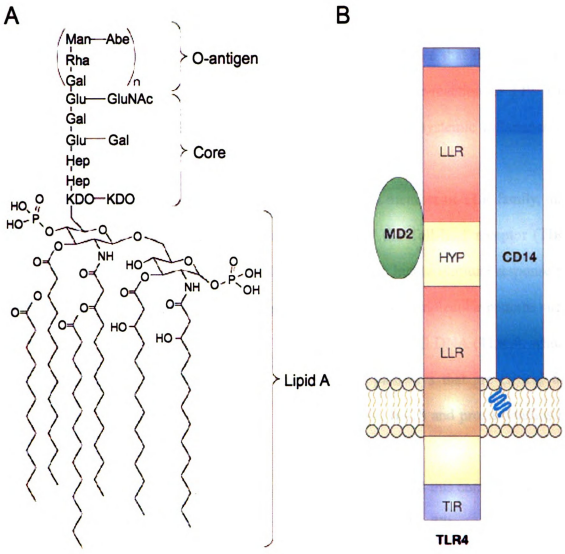


Figure 6. Chemical structure of lipopolysacchride (LPS) and a diagram of the LPS receptor. A. Bacterial LPS is composed of a hydrophobic domain known as lipid A (endotoxin), a nonrepeating core oligosaccharide, and a distal polysaccharide (O-antigen). B. Components of toll-like-receptor-4 (TLR4) complex. CD14 is a glycosylphosphatidylinositol (GPI) anchored, high-affinity membrane protein that also exists in a soluble form and concentrates LPS for binding to the TLR4-MD2 complex. LRR, leucine-rich repeats; HYP, hypervariable region. Figure adapted from Miller *et. al.* (81)

the O-specific polysaccharide provides ligands for bacterial attachment and confers some resistance to phagocytosis (82, 83). While there are many agents that can trigger the synthesis of COX-2, LPS attracts much attention because over-reaction to LPS can provoke life-threatening conditions such as septic shock or the systemic inflammatory response syndrome.

LPS is recognized by Toll-like receptor-4 (TLR4), a member of TLR family, that is defined by having a conserved cytosolic region termed the Toll-IL-1 receptor (TIR) domain (Fig. 6B) (84). TLRs are crucially involved in the innate immune response to microbes. This is accomplished by sensing pathogen-associated molecular patterns such as LPS (TLR4), peptidoglycan (TLR2), ds RNA (TLR3), and CpG DNA (TLR9), which are also inducers for COX-2. TLRs activate signaling pathways that are critical for induction of the immune response such as releasing TNF-α, IL-1β and prostaglandins in macrophage and monocyte cells (85).

LPS forms a complex with LPS-binding protein (LBP) and this complex interacts with the monocyte differentiation antigen CD14 (Fig. 7) (84, 86, 87). The binding of the LPS/LBP complex to CD14 and the TLR4-MD2 complex induces receptor dimerization. Ligand binding to the extracellular domain of TIR domain-containing receptors results in the recruitment of soluble adapter molecules including myeloid differentiation factor (MyD88), IL-1R associated kinase (IRAK), Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF; TICAM-1) and TRIF-related adaptor molecule (TRAM/TICAM2/TIRP) to its intracellular TIR domain. TLR4 signaling consists of two distinct pathways (Fig. 6 & 7). The MyD88 dependent pathway leads to the production of inflammatory cytokines, and the MyD88 independent (TRIF-dependent) pathway is associated with the stimulation of IFN-β and the maturation of dendritic cell

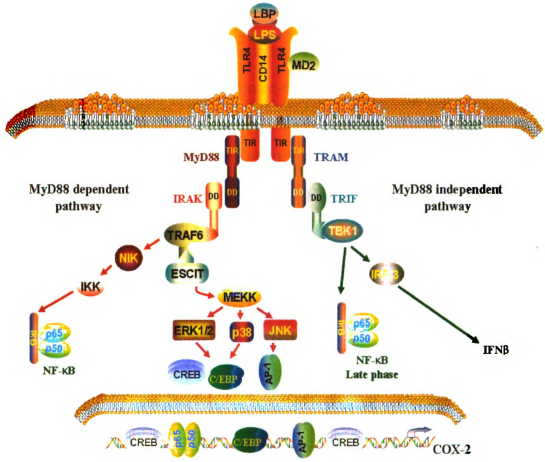


Figure 7. Toll-like receptor 4 signal transduction pathways.

There are two groups of TLR4 signaling pathways based on their use of TLR adaptors. A. MyD88 independent pathway which leads to inductioin of IFN-β, IFN-inducible genes, and maturation of dendritic cells with delayed activation of NF-κB and MAPK. B. MyD88 dependent pathway which leads to pro-inflammatory cytokine production including prostaglandins, TNF-α, IL-6, and IL-12 with early activation of NF-κB and MAPK.

(88).

MyD88 contains both a TIR domain and a death domain. When associated with a TLR, MyD88 recruits IRAK through death domain-death domain homophilic interactions. IRAK is a serine-threonine kinase involved in the phosphorylation and activation of TNF receptor associated kinase-6 (TRAF-6), and the TRAF-6 recruits evolutionarily conserved intermediate in toll (ECSIT) signaling factor (84, 87) leading to activation of a MAPK kinase kinase (MAPKKK), MEKK-1 and TGFβ-activated kinase (TAK-1). Activated MEKK-1 and activated TAK-1 phosphorylate MAPK kinases, MKK1/2, MKK4/7 and MKK3/6, which in turn, activate ERK1/2, JNK and p38 MAPKs (Fig. 5). TRAF-6 and TAK-1 also activate the IκBα kinase complex (IKK) through NF-κB inducing kinase (NIK), leading to NF-κB activation (84).

Another signaling pathway, the MyD88-independent pathway involving TRIF/TRAM adaptor proteins leads to delayed NF-κB activation (88, 89). TRIF also induces the activation of the transcriptional regulator, IFN regulatory factor (IRF)-3 and the expression of IFN-β and IFN-inducible genes through the activation of TANK-binding kinase (TBK)1 and IKKε (Fig. 7) (90). In RAW 264.7 macrophages, activated MAP kinases and NIK up-regulate COX-2 expression as well as expression of other pro-inflammatory cytokines (85).

Human U937 monocytic cells undergo morphologic and functional changes and differentiate to macrophage-like cells when treated with PMA. U937 cells do not express COX-2 mRNA or protein in the undifferentiated state, but during differentiation, low levels of COX-2 are expressed. COX-2 is further induced by inflammatory stimuli such as LPS, TNF-α or IL-1 (Table II) (64). LPS-induced COX-2 expression in U937 cells involves CRE-1, C/EBP and the downstream NF-κB elements.

Platelet microparticles (MP), formed by platelet activation, activate the COX-2 gene expression and lead to prostaglandin production in U937 cells (65). MP activates PI-3-kinase resulting in the transient activation of several PKC isoforms (PKC-β/δ/ζ/λ), ERK-1/2, p42/p44 MAPK, p38, and the sustained activation of JNK-1 as well as activation of c-Jun and Elk-1 transcription factors. Curiously, MP-induced COX-2 expression does not involve the CRE-1 motif.

Prostaglandins also play a role in complications of diabetes such as hyperglycemia, accelerated atherosclerotic and inflammatory disease, oxidant stress and glycation products (AGEs) (66, 67, 91). High glucose (HG) and AGE treatment of THP-1 monocytic cells, which are similar to U937 monocytes, lead to a significant increase in COX-2 mRNA and protein. The increase in COX-2 mRNA is predominantly due to transcriptional upregulation (67). AGEs act via RAGE (receptor for AGE) and AGEs and S100b, a specific ligand for RAGE, activate multiple signaling pathways including those involving p38, MEK/ERK, oxidant stress, PKC, and NF-κB; however, the JNK pathway is not activated in THP-1 cells in response to AGEs (91). Interestingly, AGEs and S100b induce COX-2 via the distal NF-κB site (-455/-428) while HG induces COX-2 transcription via the proximal NF-κB site (-232/-205) (66).

HG treatment of THP-1 monocytes activates PKC and the p38 MAPK pathway but not the ERK or JAK-STAT pathways. HG-induced COX-2 expression requires the CRE-1 element and activation of the CREB transcription factor as well as NF-κB activation (66). The association of CBP/p300, p/CAP (p300/CBP associated protein) and NF-κB transcription factor with the COX-2 promoter were evaluated by chromatin immunoprecipitation assays (ChIPs) with HG treated THP-1 cells (67). CBP, p/CAP, and p65 are recruited to the COX-2 promoter sequentially. p65 and CBP association

occurs after 16 hr of HG treatment, peaks after 24 hr, and decreases at 48 to 72 hr. p/CAP and p50 are recruited to the COX-2 promoter in parallel and appears as early as 16 hr after HG stimulation, increasing over time, and remaining at 72 hr. The transcriptional repressor, HDAC-1 is associated with the COX-2 promoter under basal conditions. After HG treatment, the association decreases with time as the binding of CBP increases. This suggests that recruitment of activated transcription factors such as p65 to the COX-2 promoter after HG stimulation in THP-1 monocytes is enabled by dissociation of HDAC-1 from the promoter.

The murine macrophage cell line RAW 264.7 has been used extensively as a model for examining macrophage activation and the inflammatory response. Various inflammatory mediators and cytokines, catalase, peptidoglycan (a cell wall component of gram-positive bacteria), double-stranded RNA, viral infection, and LPS stimulation cause COX-2 induction and prostaglandin formation in RAW 264.7 macrophages (14, 68-70).

It has been shown that COX-2 induction in LPS-stimulated RAW 264.7 cells consisting of an early phase of rapid induction of COX-2 mRNA expression after 1 hr of LPS-treatment followed by a phase of sustained mRNA expression (92). These authors also suggested that these different phases of mRNA expression required different sets of transcriptional activators. Consistent with this idea, the early phase of COX-2 expression was shown to be independent of *de novo* protein synthesis, whereas in the second phase, synthesis of C/EBPδ was required. Furthermore, a C/EBPβ homodimer was bound to a C/EBP element in the initial phase while a C/CBPβ·δ heterodimer bound to the C/EBP element during the second phase (92, 93). It has also been demonstrated that CREB and NF-κB are important in LPS-induced COX-2 transcription in RAW 264.7 cells. Taken together, these studies suggested that the NF-κB and C/EBP sites and the

CRE-1 are important for regulating COX-2 transcription in LPS-stimulated macrophages and that COX-2 transcription in this system consists of several phases that lead to persistent gene activation.

Using nuclear run-on assays and northern blot analyses we have shown recently that COX-2 gene transcription is rapidly increased and sustained during the 12 hr after LPS stimulation in RAW 264.7 cells (unpublished data). These findings indicate that COX-2 is mainly regulated at the transcriptional level in this system. In addition to a previously identified CRE-1, we identified a second functional cAMP response element (CRE-2) within the COX-2 proomoter. The CRE-2 is constitutively bound by CREB. On the other hand, the p65/p50 heterodimer inducibly binds to the NF-κB site after 1 hr of LPS treatment but after 4-12 hr is replaced by p50 homodimer (unpublished data). Thus, it is possible that the p65/p50 heterodimer together with CREB is required to initiate COX-2 gene transcription, and the p50 homodimer together with CREB is required to sustain the activation by interacting with a different set of transcriptional coactivators. It will be important to determine if there is a cooperative interplay between the factors bound to the CRE-2 and the NF-κB site in the COX-2 gene promoter.

We also found that an AP-1 element is required for optimal induction of COX-2 in LPS-stimulated RAW 264.7 cells. Consistent with what has been observed in PMA-treated MC3T3 cells, the CRE-1 site is constitutively bound by CREB/ATF transcription factor family members and the AP-1 element is inducibly bound by phosphorylated c-Jun and c-fos in the initial phase and by phosphorylated c-Jun dimers at the late phase of COX-2 transcription in LPS-treated RAW 264.7 macrophages (unpublished data). It is clear that COX-2 gene transcription is activated through multiple redundant mechanisms by LPS in macrophages.

3. COX protein degradation

COX-1 and COX-2 have very different rates of degradation. COX-1 is very slowly degraded whereas COX-2 protein degredation varies from 2-24 hr depending on the cell type (94). Thus, COX-1 protein is more stable even though both isoforms have very similar structures. The mechanism of COX-1 enzyme degradation has not been studied. It has been reported that COX-2 protein is ubiquitinated and degraded by the 26S proteasome in the cytoplasm in certain cell types including colon carcinoma cells and mouse neuronal cells (94-96). The 26S proteasome degrades misfolded or structurally damaged ER proteins by unfolding and translocating ubiquitinated targets into the interior of the proteasome complex (97). It is likely that both COX-1 and COX-2 enzymes can be degraded via the ER-associated degradation (ERAD) pathway involving retrograde movement of the enzymes from the ER into the cytoplasm because both isoforms are located and associated monotopically with the luminal faces of the endoplasmic reticulum membrane, the inner nuclear membrane, and the outer nuclear membrane (97-104).

The molecular basis for the differences in stabilities of COX isoforms is unknown. It is possible that COX-2 is transported into the ERAD pathway by virtue of its having specific markers that are absent from COX-1 (105, 106). As noted earlier, the most obvious structural difference between COX-1 and COX-2 is the 18 amino acids cassette unique to COX-2 (Fig.1).

In addition, the binding of nonsteriodal anti-inflammatory drugs (NSAIDs) or COX-2 inhibitors retards COX-2 but not COX-1 protein degradation (unpublished data). This stabilization of COX-2 is consistent with formation of a more compact, ERAD-resistant form of the enzyme. Alternatively, NSAIDs may protect COX-2 by preventing

catalysis.

Concluding Remarks

Regulation of COX gene expression is a complex process that varies in different cell types and even between the same cell types in different species. COX-1 and COX-2 genes are activated by a wide variety of stimuli acting through numerous signaling pathways and the relative contribution of each depends upon the stimulus, the cell type, and the time of stimulation. These factors and conditions determine which transcription factors are associated with the COX response elements. Although we now have a general understanding of the factors involved in COX-2 gene regulation, the interplay between the various regulatory transcription factors remains to be elucidated.

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CHAPTER 2

CYCLOOXYGENASE-2 GENE TRANSCRIPTION IN A MACROPHAGE MODEL OF INFLAMMATION

Summary

Infections involving lipopolysaccharide (LPS)-bearing, gram-negative bacteria can lead to acute inflammation and septic shock. Cyclooxygenase-2 (COX-2), the target of nonsteroidal anti-inflammatory drugs and selective COX-2 inhibitors, is importantly involved in these immunological responses. We examined the dynamics of COX-2 gene expression in RAW264.7 murine macrophages treated with LPS as a model for COX-2 gene expression during inflammation. We first established using Northern blotting and nuclear run-on assays that COX-2 transcriptional activation continues for at least 12 hr after LPS treatment and involves at least three phases. Previous studies with murine macrophages identified an NF-κB site, a C/EBP site, and a CRE-1 as *cis*-acting elements in the COX-2 promoter. We identified three additional functional elements including a second CRE (CRE-2), an AP-1 site, and an E-box that overlaps the CRE-1. The E-box mediates transcriptional repression whereas the other *cis*-elements are activating. Using electrophoretic mobility supershift assays and chromatin immunoprecipitation assays, we cataloged binding to each functional *cis*-element and found them to be occupied to varying extents and by different transcription factors during the 12 hrs following LPS treatment. This suggests that the *cis*-elements and their cognate transcription factors participate in a sequential, coordinated regulation of COX-2 gene expression during an inflammatory response. In support of this concept, we found using inhibitors of Jun

kinase and NF-κB p50 nuclear localization that COX-2 gene transcription was completely dependent on phospho-c-Jun plus p50 at six hr after LPS treatment but only partially dependent on the combination of these factors at later treatment times.

Introduction

Prostaglandin endoperoxide H synthases, commonly known as "cyclooxygenases," (COX) catalyze the committed step in the conversion of arachidonic acid to prostaglandins (1, 2). There are two COX isoforms, the predominantly constitutive isoform, COX-1, and an inducible isoform, COX-2. Although both enzymes catalyze the same reaction with similar kinetics *in vitro*, *in vivo* studies with isoform specific inhibitors and COX-1 and COX-2 knock out mice indicate that there are some physiological processes that require one specific enzyme and others where the isoforms can complement one another (3, 4).

Bacterial lipopolysaccharide (LPS) is a potent inducer of COX-2 expression in macrophage cells. This induction is a result of LPS activation of the toll-like receptor-4 (TLR4), which, in turn, initiates signaling through MyD88/IRAK/TRAF6/ECSIT resulting in the activation of ERK, JNK, p38, PKC, and NIK (5, 6). These kinases exert their actions by phosphorylating either transcription factors or other downstream effectors to cause the transcriptional machinery to begin transcribing the COX-2 gene. LPS-induced COX-2 transcription is regulated through multiple redundant mechanisms involving several central response elements present in the COX-2 promoter (6, 7). The CRE-1 at –57/-52 in the murine COX-2 promoter is necessary for mediating the effects of a wide variety of stimuli, while a C/EBP site and an NF-κB response element appear to function in more specialized signaling events.

The promoters of the human, murine, rat, equine, and bovine COX-2 genes have a number of common putative regulatory elements. In the mouse promoter, there are both a cAMP response element (CRE-2) and an NF-κB site located between 400 and 550 bp

upstream of the transcription start site, a C/EBP site at -138/-130, an AP-1 site at -73/-61 and an overlapping CRE-1/E-box element at -59/-48 (Fig. 1). The NF-κB site is necessary for inducible COX-2 promoter activity in TNF-α stimulated MC3T3-E1 cells (8). More recently, Hwang *et al.* have demonstrated that blocking NF-κB activation at several levels results in a large decrease in COX-2 promoter activity in RAW 264.7 cells (6, 9). The CRE-2 site has previously been tested in fluid sheer stress stimulated osteoblastic MC3T3-E1 cells, but a mutation in this site had no effect on the transcriptional activation of a COX-2 promoter reporter gene in this system (10). However, the CRE-2 site has been demonstrated to play an important role in IL-1β-induced COX-2 transcription in the endometrium (11). MC3T3-E1 osteoblasts cells treated with PMA activate COX-2 transcription in a process mediated by the AP-1 site (12, 13). The overlapping CRE-1 and E-box element of the human promoter have been studied in transfected LPS-stimulated RAW 264.7 macrophages (14) and shown to be involved in stimulation of COX-2 transcription. The E-box element has also been shown to act as a positive regulatory element for COX-2 expression in granulosa cells (15) and in PMA-treated human gastric epithelial cells (16).

Unlike fibroblasts where COX-2 is only transiently expressed (17), monocytes/macrophages express COX-2 for a prolonged time (18). Although the transcriptional regulation of COX-2 has been extensively studied, little is known about the dynamics of the essential *cis*-acting elements of COX-2 promoter during transcriptional activation and the mechanism of sustained activation. In this report we demonstrate the involvement of the CRE-1, CRE-2, NF-κB, and AP-1 sites in positive COX-2 transcriptional regulation and provide the first evidence of negative regulation of

COX-2 gene transcription through the E-box element in LPS-treated RAW 264.7 macrophages. We have also identified nuclear proteins associated with these response elements over a 12 hr time period following LPS treatment and established that c-Jun phosphorylation and p50 nuclear translocation are uniquely required in the middle phase of COX-2 transcription. Cumulatively, our data demonstrate that the COX-2 gene is transcriptionally regulated in LPS-treated RAW 264.7 macrophages in a coordinated manner by unique pairings of transcription factors and promoter elements to sustain COX-2 transcriptional activation for 12 hr after initiating LPS treatment.

Materials and Methods

Reagents and antibodies. Lipopolysaccharide (LPS) from *Salmonella minnesota* was purchased from Sigma-Aldrich Chemical Co. Rabbit anti-p50 and anti-p65 antibodies were kindly provided by N. Rice (NCI-Frederick) and CREB-1, ATF, USF-1, and USF-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), antibody to phospho-CREB was purchased from Upstate Biotechnology (Lake Placid, NY) and antibodies to CREB and CBP were purchased from Cell Signaling (Beverly, MA). Complete™ Protease Inhibitor Cocktail and Pefabloc were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Poly(dI-dC)-Poly(dI-dC) (average length 8517 bp) was purchased from Pharmacia Biotech. All other chemicals and reagents were purchased from J.T. Baker (Phillipsburg, NJ). Plasmid DNA was isolated with Qiagen Endo-Free™ Maxi-prep columns. Nuclei for electrophoretic mobility shift assays (EMSAs) were isolated with NE-PER™ nuclear and cytoplasmic extraction reagents from Pierce Biochemical Co. (Rockford, IL). JNK inhibitor and p50 inhibitor were purchased from Calbiochem.

Plasmids. The various regions of the murine COX-2 promoter were cloned into pGL3basic (Promega). The -966/+23 construct was cloned from a KpnI and HindIII fragment (19). Mutagenesis was performed using the Stratagene Quick Change protocol with *pfu* Turbo DNA polymerase (Stratagene). The sequences of the mutant and promoter constructs were verified; the mutations are summarized in Table III. pRC-p50 expression plasmid was kindly provided by Professor Richard Schwartz (Michigan State University), and the mCOX-2 plasmid, pSVLN-muCOX-2, was provided by Professor David L. DeWitt (Michigan State University). pRSV-ACREB, pRSV-IκB S/R, and

pET23b-p300 expression plasmids were kindly provided by Professor Roland Kwok (University of Michigan). The pcDNA3-USF-1/-2 expression vectors were generated by cloning HindIII-XbaI-flanked PCR products derived from USF-1/USF-2 cDNA into the Hind III-XbaI-digested pcDNA3 vector (Invitrogen).

Cell culture and transfections. RAW 264.7 cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and Gentamicin (100 μg /ml) (Life Technologies, Inc.) and were maintained at 37° C in 5% CO_2. RAW 264.7 cells were cultured in six well plates at a density of 5 x 10^5 cells/ml one day prior to transfection. A luciferase reporter plasmid (2.5 μg) and a pCMV-β-galactosidase plasmid (0.5 μg; Pharmacia) were transfected into RAW 264.7 cells for 45 min using DEAE-dextran (400 μg/ml) and 100 mM Tris-HCl, pH 7.3, in 600 ml of DMEM. The transfection reaction mixture was removed, and the cells were cultured in DMEM with 10% FBS for 12 to 24 hr before LPS (200 ng/ml) stimulation for 12 hr. For transfections using Superfect (Qiagen), 5 x 10^5 cells/well were plated 24 hr prior to transfection. COX-2 (1 μg) plasmid and pSV-β-galactosidase (0.5 μg) plasmids (Promega) were transfected into RAW 264.7 cells according to manufacture's instruction.

Luciferase reporter assays. Chemiluminescent luciferase activity assays were performed using reagents from Promega and a Molecular Dynamics luminometer. β-galactosidase activity was measured using an ONPG assay (Invitrogen) per the instructions of the manufacturer. Protein concentrations were determined using the

Bradford reagent (BioRad). Spectrophotometric measurements were made on a Molecular Dynamics 96 well plate reader. Luciferase activity is represented as relative luciferase units of firefly per β-galactosidase activity. Statistically significant differences between groups versus control were obtained with a Student t test, and significant differences are indicated by asterisks.

Western blot analysis. RAW 264.7 cells were treated without or with LPS for various times (1, 2, 4, 6, 8, 10, or 12 hr) and lysed in 1% SDS lysis buffer. Protein concentration is measured and 20 μg of protein for each sample is loaded to SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was incubated with COX-2 or actin antibodies and visualized with an enhanced chemiluminescence system.

Northern blot analysis. Total cellular RNA was isolated from cells using Trizol RNA isolation reagent (Life Technologies). Total cellular RNA (15 μg) was electophoresed on a 3.7% formaldehyde, 0.8% agarose gel in TAE (10 mM Tris-acetate, pH 8 to 8.1, 10 mM EDTA) and transferred to a nitrocellulose membrane. The membrane was pre-hybridized for 1 hr at 65° C in pre-hybridization buffer (5x SSC, 50% formamide, 5x Denhardt's, 1% SDS, and sheared salmon sperm DNA (100 μg/ml)) and hybridized with a probe in TES/NaCl Solution (10 mM TrisHCl, pH 7.4, 10 mM EDTA, 0.2% SDS, and 0.6 M NaCl). Following hybridization, the membranes were washed twice in 2x SSC at 65°C for 20 min. A 1.8 kb NotI fragment of pSVLN mCOX-2 and a 1.3 kb EcoR1, XhoI fragment of a β-plasmid (Stratagene) were labeled by random priming using a Mega Prime Labeling kit (Amersham) with α-^{32}P-CTP (New England

Nuclear). The membranes were exposed to a phosphoimaging screen, and densitometry was performed using Image Quant Software.

Electrophoretic mobility shift assays. Nuclei were isolated from RAW 264.7 cells that had been stimulated for 1 or 12 hr with LPS (200 ng/ml). Cell lysis and nuclear isolations were performed using NE-PER™ reagents (Pierce) per the instructions of the manufacturer in the presence of 2 mM Pefabloc and 1X Complete Protease Inhibitor Cocktail. Oligonucleotide probes (Michigan State University Macro-molecular Structure Facility and Invitrogen) were annealed in T4 PNK Buffer and end-labeled with T4 Polynucleotide Kinase (New England Biological) and γ32P-ATP (New England Nuclear). The sequences of the probes are summarized in Table III. The probes were electrophoresed on a TAE 10% acrylamide gel. The double stranded probes were excised and eluted for 2 hr at 37° C in 0.5 M sodium acetate, 10 mM MgCl2, 1 mM EDTA and 0.1% SDS, ethanol precipitated and resuspended in TE. Binding reactions were performed with nuclear extract (5 μg of protein) and probe in the presence of 100 mM KCl, 20 mM HEPES, pH 7.9, 1 mM EDTA, 10% glycerol, 2 mM Pefabloc, 1X Complete Protease Inhibitor Cocktail, 2 mM DTT and 2 μg of Poly(dI-dC)-Poly(dI-dC) and electrophoresed on 5% TBE acrylamide gels at 150 VDC for 1.5 hr. For EMSAs, the probes were incubated with the nuclear extracts for 1 hr at 25° C, and for supershift assays, the nuclear extracts were combined with the probes for 15 min at 25° C and then incubated with antisera for 4-6 hr at 2-8° C. EMSA gels were dried on 3M filter paper and exposed to a phosphoimaging screen, and densitometry was performed using Image Quant software (Molecular Dynamics).

Nuclear run-on assays. RAW 264.7 cells (ca. 10^7) were stimulated with LPS (200 ng/ml) for 0.25, 0.5, 1, 3, 6, 9 or 12 hr. The cells were rinsed twice with ice cold PBS and were scraped into 1 ml of PBS. The cells were collected by centrifugation and lysed by resuspension in 1 ml of 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% IGEPAL CA-650 (Sigma) for 5 min. The nuclei were collected by centrifugation for 10 min at 1000 rpm in a mini-centrifuge and resuspended in 500 µl of Freeze Buffer (50 mM Tris-HCl, pH 8.3, glycerol (40% v/v), 5 mM $MgCl_2$, and 0.1 mM EDTA). Nuclei were either used fresh or stored at –80° C. To begin a run-on reaction, 225 µl of suspended nuclei (ca. 10^7) was combined with 60 µl of Run-On Buffer (25 mM Tris-HCl, pH 8.0, 12.5 mM $MgCl_2$, 750 mM KCl, 1.25 mM ATP, GTP, CTP, 2 mM DTT), 120 units of RNase-OUT (Life Technologies), and 100 µCi of α-^{32}P-UTP. After 15 min at 37° C, 1 ml of Trizol was added, followed by 200 µl of chloroform. The mixture was vortexed vigorously and transferred to pre-spun Phase Lock Gel tube (Beckman/Eppindorf) and centrifuged for 10 min at 10,000 x g. The aqueous phase was removed and combined with 500 µl of isopropanol. The RNA was precipitated at –80° C for 15 min then centrifuged for 20 min at 10,000 x g and resuspended in RNase-free H_2O. The RNA was further purified and genomic DNA was fragmented with RNase-free DNase I (Life Technologies) at 37° C for 5 min, then chilled on ice for 5 min before the addition of 1 M NaOH for exactly 2 min, followed by the addition of 1 M HEPES (free acid). The RNA was then precipitated by the addition of isopropanol. After centrifugation the pellets were resuspended in DEPC treated water. The activity was determined by scintillation counting. Slot blots were prepared on nitrocellulose

membranes (Schleicher and Schuell) with 10 μg of denatured, linearized murine COX-2 expression plasmid (pSVLN-mCOX-2), β-plasmid (Stratagene), empty vector, or vehicle without plasmid. After baking, the membranes were pre-hybridized in 5x SSC, 50% formamide, 5x Denhardt's solution, 1% SDS, and sheared salmon sperm DNA (100 μg/ml) for 1 hr at 65° C. Hybridization of the labeled RNAs with membranes was performed at 65 °C in TES/NaCl Solution (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.2% SDS, and 0.6 M NaCl). Following hybridization the membranes were rinsed twice in 10x SSC, then washed twice in 2x SSC at 65° C for 20 min. The dried membranes were exposed to a phosphoimaging screen, and densitometry was performed using Image Quant Software (Molecular Dynamics).

Reverse transcriptase-PCR. RAW 264.7 cells were treated with 200 ng/ml of LPS for various times and total RNA was isolated using RNAeasy kit (Quagen). For JNK inhibitor and p50 inhibitor studies, 10 μM of JNK inhibitor or 90 μM of p50 inhibitor was added after 3.5 hr of LPS treatment. The RNA (2 μg) was treated with DNase I and 8 μl of each sample was reverse-transcribed and amplified using one-step RT-PCR system (invitrogen) for 35 cycles with specific primers for COX-2 mRNA and actin control nRNA. The one step RT-PCR cycle parameters were 45 °C for 30 min, 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 1 min. The primers used for COX-2 mRNA: forward primer ACACTCTATCACTGGCATCC; reverse primer GAAGGGACACCCTTTCACAT, primers for actin mRNA: forward primer CACACCCGCCACCAGTTC; reverse primer ACGCACGATTTCCCTCTCA. PCR

products were electrophoresed on 1% agarose gel and visualized by staining with cybergreen.

Chromatin immunoprecipitation assay. LPS treated RAW 264.7 cells were treated with 1% aqueous formaldehyde at room temperature for 20 min. Cells were washed twice with 10 ml of ice cold PBS and scraped into 10 ml conical tubes. The suspended cells were then washed with 10 ml of ice cold Buffer A (0.25% NP-40, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5, 0.5 mM PMSF) and Buffer B (20 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5, 0.5 mM PMSF) and lysed with a lysis buffer (1% SDS, 10 mM DETA, 50 mM Tris-HCl, pH 8, 1X complete protease inhibitor cocktail, 2 mM perfobloc); 1ml of the lysis buffer was used for per 10^8 cells. Cell lysates were sonicated seven times for 15 sec each at setting 3 on a Misonix sonicator to shear the DNA to lengths between 200 and 1000 bp. Samples were then centrifuged for 10 min at 12,000 rpm at 4° C. The supernatant fraction was diluted ten fold in dilution buffer (1% Triton-X 100, 2 mM EDTA, 150 mM NaCl, 20 mM TrisHCl, pH 8, 1X complete protease inhibitor cocktail, 2mM perfobloc) and 2 µg of p50, p65, CREB-1, c-Jun, p300, USF-1, or USF-2 antibody was added for each immunoprecipitation. After an overnight incubation with the antibody, 20 µl of 50 % slurry of Protein A/G agarose, which had been washed three times with dilution buffer was added to the sample and the samples incubated for four hr at 4 °C with gentle mixing. Immunoprecipitated materials were washed 1 time with Buffer TSE (0.1% SDS, 1% TritonX100, 2 mM EDTA, 20 mM Tris pH 8.0), 1 time with Buffer TSE+250 mM NaCl, 1 time with Buffer TSE+500 mM NaCl, 1 time with Buffer C (0.25 M LiCl, 1% NP-40,

1% DOC, 1 mM EDTA, 10 mM Tri-HCl, pH 8), and 3 times with TE Buffer. Protein-DNA complexes were eluted with 300 µl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 30 min at room temperature. NaCl (200 mM) was added, and the samples were incubated at 65 °C for 16 hr. After reverse cross-linking, 3 µl of protease K (20 mg/ml) and 1 µl of an aqueous glycogen solution (20 mg/ml) were added, and the samples were incubated at 42 °C for 2 hr. DNA was extracted with phenol/chloroform. The pellet was resuspended in 40 µl of water. For PCR reactions, 5 µl of each sample was used and 20 µl of the 50 µl PCR product was loaded onto agarose gels. To obtain conditions where the signal intensity was linear with the input, various numbers of cycles of PCR were performed; 28 cycles (26 cycles for CBP ChIP assay) of reaction were used in the experiments depicted in the figures. PCR primers were as follows: for GAPDH: 5'-GCTGACATCAACTCCCAGGT-3', 5'-TTCCGTTCTCAGCCTTGACT-3', for COX-2 (distal region): 5'-TCCCGGGATCTAAGGTCCTA-3', 5'CAGATGTGGACCCTGACAGA-3', for COX-2 (proximal region): 5'-TCCTTCGTGAGCAGAGTCCT-3', 5'-CGCAACTCACTGAAGCAGAG-3'. PCR products were electrophoresed on 2% agarose gels and visualized by staining with cybergreen. PCR products for GAPDH, COX-2 (distal region) and COX-2 (proximal region) are 470 bp, 159 bp and 248 bp respectively.

Results

LPS causes sustained increases in the rate of COX-2 gene transcription in RAW 264.7 cells. Stimulation of macrophage and macrophage-like cells with LPS results in the synthesis of prostaglandins and sustained increases in the expression of COX-2 protein and other inflammation-related proteins (Fig. 9A) (6, 20-23). It has been hypothesized that the sustained increase in COX-2 expression is a result of increased transcription of the COX-2 gene, but there has been no direct demonstration of this. Using nuclear run-on assays we established that LPS treatment of RAW 264.7 cells does cause a rapid and prolonged increase in the rate of synthesis of COX-2 mRNA (Fig. 9)(24). Furthermore, the rate of mRNA synthesis correlates temporally with the amounts of COX-2 mRNA extracted from RAW 264.7 cells at various times before and after LPS treatment. A decrease or pause in the rate of COX-2 mRNA synthesis and mRNA accumulation was consistently seen between 4-8 hr after initiating LPS treatment in all of the nuclear run-on assays and Northern blot time courses that were performed during our studies. This suggests that LPS-induced COX-2 gene expression in this model system has early (ca. 0-4 hr), middle (ca. 4-8 hr) and late (ca. 8-12 hr) phases. Further support for this is presented at the end of the Results section.

Characterization of new cis-acting elements in the murine mCOX-2 promoter associated with LPS-induced COX-2 gene expression. Several cis-acting elements in the mCOX-2 promoter, including an NF-κB site at –401/-393, a C/EBP response element at –138/-130, and a CRE-1 at –59/-52, have been shown previously to be required for LPS-induced COX-2 transcription in RAW 64.7 cells (25-27). To further

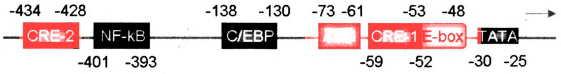


Figure 8. Schematic representation of the major conserved response elements in the murine COX-2 promoter.
Cis-acting elements found within the COX-2 promoter are noted in the shaded boxes and their location relative to the COX-2 transcriptional start site are noted above or below each element. CRE, cAMP response element; NF-κB, Nuclear factor kappa B; C/EBP, CCAAT/enhancer-binding proteins; AP, Activator protein

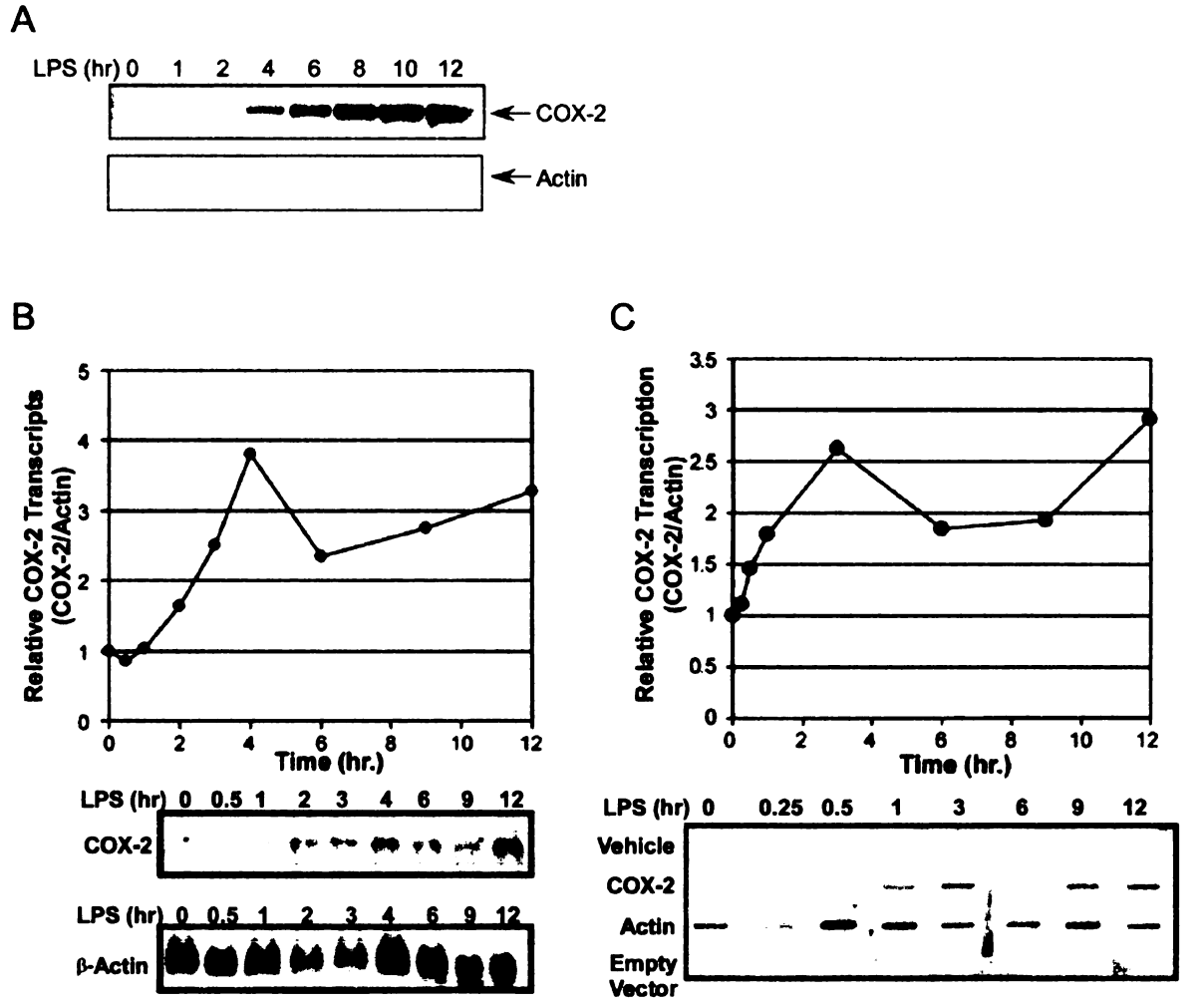


Figure 9. Sustained increase of mCOX-2 gene expression in LPS-stimulated RAW 264.7 cells. A. RAW 264.7 cells were treated with LPS (200 ng/ml) for the indicated times and cell lysates were prepared. Western blot analysis was performed with COX-2 or actin antibody as indicated with the arrows. B. Northern blot analysis was performed with RNA isolated from RAW 264.7 cells that had been treated with LPS (200 ng/ml) for the indicated times. Total RNA (15 μg) was separated on a 0.8% agarose, 4% formaldehyde gel, transferred to a nitrocellulose membrane and hybridized to COX-2 or β-actin probes as detailed in the Methods section. Similar results were obtained in five independent experiments. C. Nuclei were isolated from RAW 264.7 cells stimulated with LPS (200 ng/ml) for the indicated times and incubated with γ-^{32}P-UTP to label newly synthesized RNA transcripts. RNA was isolated and blotted onto a nitrocellulose membrane with vehicle, COX-2 cDNA, β-actin cDNA or an empty vector DNA control. Similar results were obtained in two separate experiments with two different cell preparations. B and C are obtained from wingerd *et. al.* (24).

elucidate the transcriptional regulatory components of the mCOX-2 promoter, deletion constructs of the promoter driving a luciferase reporter were transfected into RAW 264.7 cells (Fig. 10A) (24). Transfected cells were treated for 12 hr with or without LPS to determine which general regions of the mCOX-2 promoter are necessary for maximal LPS responses (Figs. 8, 10). Deletion of a segment from –459 to –414 removes a previously uncharacterized CRE (CRE-2) without eliminating the adjoining, conserved NF-κB response element (Fig. 8). Deletion of this CRE-2 site resulted in an approximately 50% reduction in the LPS-induced COX-2 expression (Fig. 10A). Larger deletions that removed the NF-κB and C/EBP-1 response elements caused no further decreases in the LPS-induced promoter responses, suggesting that the CRE-2 site is an important regulatory component of LPS mediated COX-2 induction. Additional deletion analyses of proximal regions of murine COX-2 were not performed in this study, as this region had been characterized previously in LPS-treated RAW 264.7 cells and shown to be important in regulating COX-2 mRNA accumulation (25, 28).

To investigate the relative contribution of the CRE-2 and other putative cis-acting elements of the COX-2 promoter, we engineered and analyzed mutations in the CRE-1 (-59/-52), E-box (-53/-48), AP-1 (-73/-61), CRE-2 (-434/-428), and c-Ets (-312/-306) sites in the mCOX-2 promoter (–966 to +23) fused to a luciferase reporter gene (Table III; Fig. 10B). Consistent with the deletion analysis (Fig. 10A), mutation of the CRE-2 resulted in a loss of about half of the LPS stimulated luciferase activity. The AP-1 and CRE-1 mutations eliminated approximately 75% of the reporter activity, whereas the c-Ets mutation did not cause any change in luciferase activity when compared with the wild type promoter (Fig. 10, and data not shown). Our data indicate that in addition to the

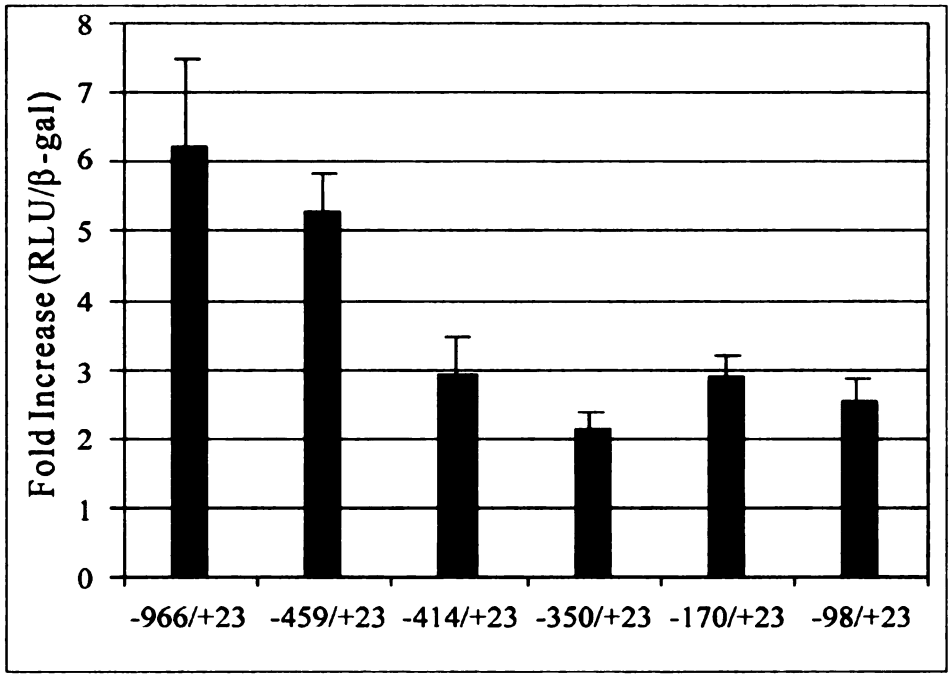
Table III. Oligonucleotides used for EMSAs and preparation of COX-2 promoter mutations.

Sequences of the primers used for EMSA probes and the generation of mutant COX-2 promoter reporter constructs. Mutated bases are denoted in lower case letters. Both the sense and anti-sense primers were used in EMSAs and mutagenesis.

Oligonucleotide	**Sequence**
CRE-2	5'-GAGCAGCGAGCACGTCAGACTGCGCC-3'
Mutant CRE-2	5'-GAGCAGCGAGCAatTCAGACTGCGCC-3'
NF-κB	5'-GAGAGGTGAGGGGATTCCCTTAGTTAG-3'
Mutant NF-κB	5'-GAGAGGTGAGGGccTTCCCTTAGTTAG-3'
AP-1	5'-CGCTTGATGACTCAGCCGGAA-3'
Mutant AP-1	5'-CGCTTGATGACTtgGCCGGAA-3'
CRE-1/ E-box	5'-TCACCACTACGTCACGTGGAGTCCG-3'
Mutant CRE-1/ E-box	5'-TCACCACTAatTCACGTGGAGTCCG-3'
CRE-1/ Mutant E-box	5'-TCACCACTACGTCAatTGGAGTCCG-3'
c-Ets	5'-TGCGCGACTGGGAGGAAACCGGAGACCC-3'
Mutant cEts	5'-TGCGCGACTGGGAGgaAACCGGAGACCC-3'

Figure 10. Promoter activity of COX-2 deletion and mutation constructs.
A. A nested set of deletions were prepared from the native –966/+23 murine COX-2 promoter. RAW 264.7 cells were transiently co-transfected with 2.5 μg of luciferase promoter reporter plasmids containing the indicated regions of the COX-2 promoter and 0.5 μg of a CMV β-galactosidase expressing plasmid. Twenty-four hr post transfection the cells were stimulated for 12 hr with LPS (200 ng/ml). Measurements of relative light intensities from the luciferase activity (Relative Luciferase Units, RLU) were normalized to the units of β-galactosidase activity as described in the Methods section. Bars represent the average fold increase in promoter activity between unstimulated control and LPS-stimulated RAW 264.7 cells for each of the COX-2 promoter reporter plasmid. The error bars represent the standard deviation relative to the average fold increase in promoter activity observed across three independent experiments. Adopted from Wingerd *et.al.* (24).
B. Reporter activities of mCOX-2 promoter reporter constructs with individual *cis*-elements mutated. RAW 264.7 cells were transiently co-transfected with 3 μg of luciferase promoter-reporter plasmids containing mutations in the indicated elements of the mCOX-2 promoter (-966/+23) and 0.5 μg of a pSV β-galactosidase expression plasmid. LPS treatment and quantification of transfection data were performed as above. Data represent the promoter activity from cells transfected with reporters driven by either the wild type COX-2 promoter or promoters with the indicated mutations, normalized to the β-galactosidase activity, with and without LPS treatment. Error bars represent the standard deviation from the average values obtained from three independent experiments performed in duplicate. *$P<0.05$ and **$P<0.01$ for native (WT) plasmid-transfected LPS-treated cells vs. mutants plasmid-transfected LPS-treated cells.

A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8
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0
-966/+23
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-350/+23
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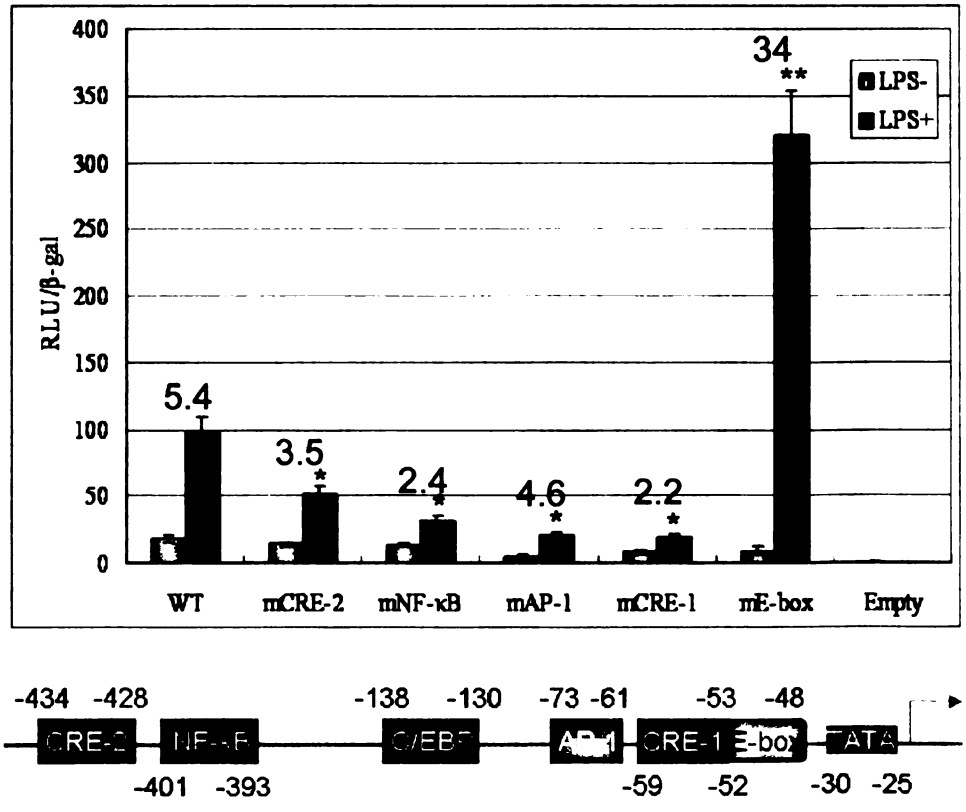
B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200
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LPS-
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34
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WT
mCRE-2
mNF-κB
mAP-1
mCRE-1
mE-box
Empty
-434
-428
-401
-393
-138
-130
-73
-61
-59
-53
-52
-48
-30
-25
CRE-2
NF-κB
C/EBP
AP-1
CRE-1
E-box
TATA

previously reported cis-acting elements (i.e. NF-κB, C/EBP, and CRE-1), the CRE-2 and the AP-1 elements are required for maximal mCOX-2 promoter activity elicited by LPS treatment of RAW 264.7 cells. Interestingly and in contrast to the reduced activity resulting from mutation of the other regulatory elements of COX-2 promoter, mutation of the E-box caused a significant increase in luciferase activity (Fig. 10B), suggesting that the E-box is involved in inhibition of mCOX-2 gene transcription in LPS-stimulated RAW 264.7 macrophages.

Transcription factor binding to *cis*-acting elements in the mCOX-2 promoter. EMSA supershift assays were performed to identify proteins binding to the COX-2 *cis*-acting elements. To examine binding to the CRE-2, a ^{32}P-labeled 26 bp double-stranded DNA probe containing the CRE-2 (5'-ACGTCA-3') (Table III) was incubated with nuclear extracts from either control RAW 264.7 cells or cells that had been treated with LPS for 0, 1 or 12 hr (Figs. 11A and 11B, lanes 1-3). The mobility and intensities of the radioactive CRE-2 protein complexes with control IgG were similar when using nuclear extracts from both treated and untreated cells. That is, the amount of CRE-2 bound by nuclear proteins does not appear to be substantially up or down regulated by LPS. Supershift assays with antibodies against CREB, ATF-1, ATF-2, or phospho-c-Jun (Figs. 11A and 11B) indicated that CRE-2 is bound constitutively by members of the CREB/ATF transcription factor family (i.e. CREB and ATF-2) and by phosphorylated c-Jun.

The NF-κB response element (5'-GGGGATTCCC-3'; Table III) is necessary for maximal mCOX-2 promoter activity in RAW 264.7 cells (5, 29), but the nuclear proteins

bound to this response element had not been characterized (Fig. 11C). p65 and p50 are known to be involved in distinct dimeric NF-κB complexes (30). To determine the proteins bound to the NF-κB site, supershift assays were performed using nuclear proteins from untreated cells or cells treated with LPS for either 1 or 12 hrs. Two distinct complexes were observed with the NF-κB probe and these nuclear extracts. Anti-p50 antibody caused shifts in both the high and low mobility complexes, whereas the anti-p65 antibody shifted only the low mobility complex (Fig. 11C). This indicates that the low mobility complex includes a p50/p65 heterodimer while the high mobility complex includes a p50/p50 homodimer. Interestingly, our data revealed that the NF-κB site is primarily occupied by the p65/p50 heterodimer during the early phase (1 hr) of the LPS response and predominantly occupied by the p50 homodimer at the late phase (12 hr) of COX-2 transcriptional activation (Fig. 11C). It was after four hrs of exposure of RAW 264.7 cells to LPS that we observed a shift in the composition of the nuclear complex bound to the NF-κB site from predominantly p50/p65 heterodimers to predominantly p50 homodimers (data not shown). This result is despite the fact that COX-2 transcription is up-regulated for at least 12 hr by LPS treatment (Fig. 9) and that the p50 homodimer is typically associated with inhibition of gene expression.

NF-κB and CRE-2 sites seemed to function coordinatedly in LPS-induced COX-2 gene expression as determined using promoter assay (24). This raises the possibility that there are protein-protein interactions linking CREB/ATF binding at the CRE-2 with p65/p50 heterodimer and/or p50 homodimer binding at the NF-κB site. To investigate transcription co-factors that are associated with this region, a 57 bp double stranded oligonucleotide probe containing both the CRE-2 and the NF-κB site was used for EMSA

(Fig. 12). Nuclear proteins bound to the 57 bp probe to form several complexes (Fig. 12, lane 1-3). To discriminate among these radioactive bands, 100X cold probe competitors (Fig. 12A) or ^{32}P-labeled probes (Fig. 12B) containing mutations of either CRE-2 or NF-κB site or both were used. Mutant CRE-2 cold probe that competes NF-κB site binding (Fig. 12A, lane 4-6) and ^{32}P-labeled mutant NF-κB probe (Fig. 12B, lane 7-9) were able to eliminate the shifts caused by p65/p50 heterodimers and p50 homodimers, and there are no additional bands that represent CRE-2 complex when compared with WT cold probe, which competes for both CRE-2 and NF-κB binding (Fig. 12A, lane 13-15) and ^{32}P-labeled double mutant (Fig. 12B, lane 13-15).

To identify transcription factors bound to this 57 bp probe, supershift assays were performed using antibodies to p65, phosphorylated p65, p50, or CREB was performed (Fig. 12C). p50 antibody was be able to shift the entire complex (Fig. 12C, lane 10-12) whereas anti-CREB antibody caused only very faint shifts in bands (Fig. 12C, lane, 4-6), indicating that CREB is bound to 57 bp probe even though CRE-2 complex was difficult to detect in EMSAs (Fig. 12A,B). However, phosphorylated p65 and p65 supershifts were not observed, which could be observed if higher quality antibodies were used.

Another way to characterize transcription factor binding to the upstream region of COX-2 after LPS stimulation is to use biotin-streptavidin pull down assays. Briefly, biotin-labeled probe is incubated with nuclear extract, and the complex formed is immobilized with streptavidin-coated beads. The beads are washed and proteins were eluted and immunoblotted with antibodies against different transcription factors. Because a CRE could be bound by members of the AP-1 transcription factor family as

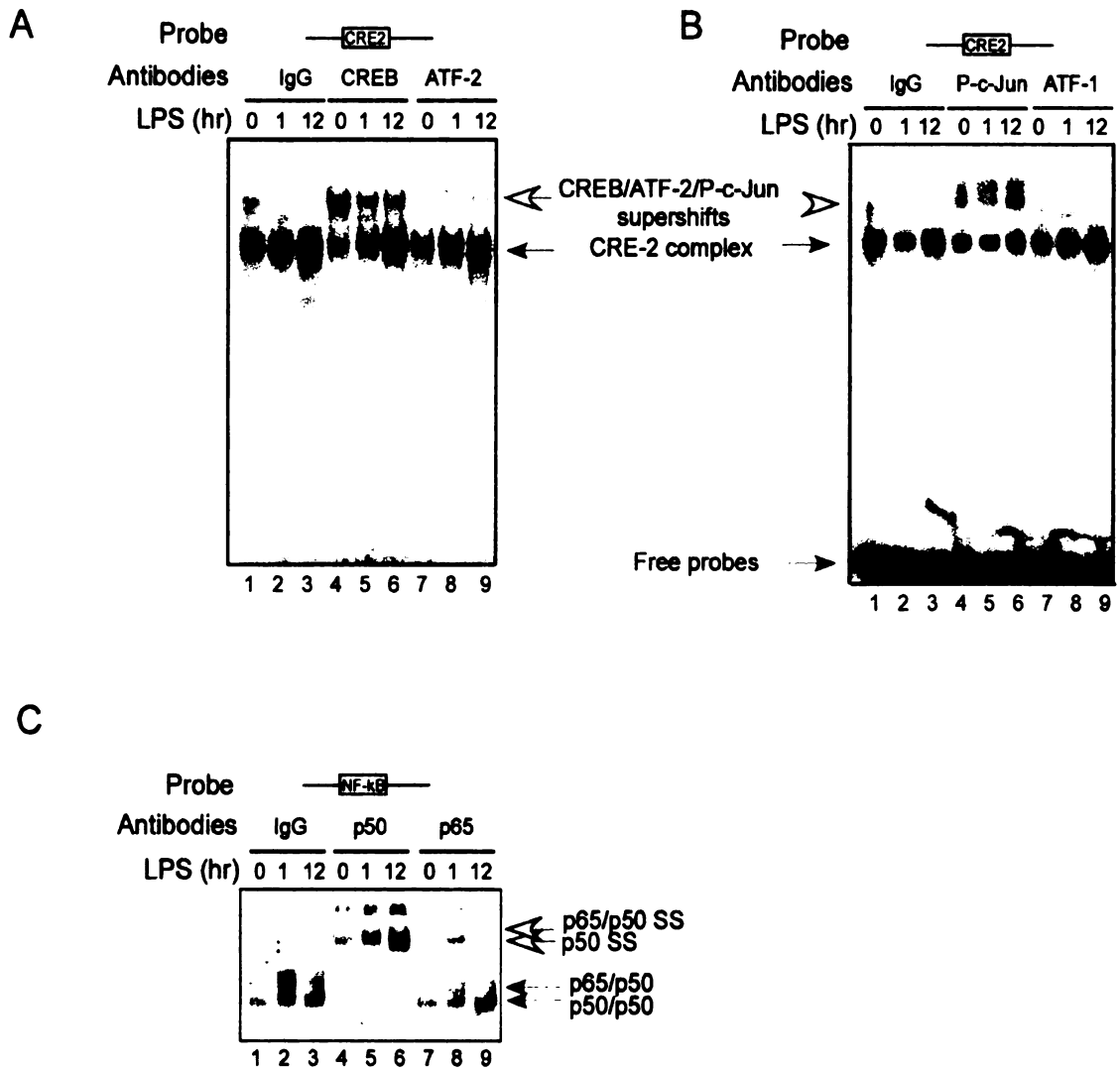
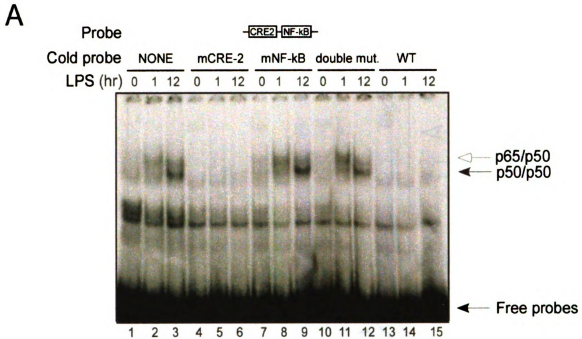
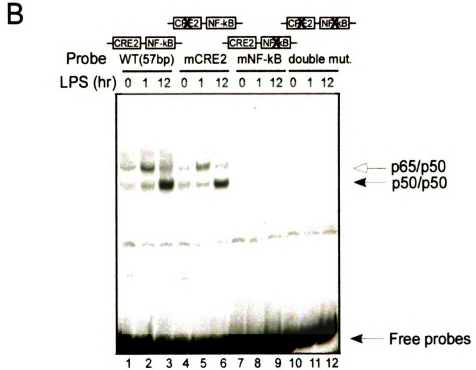


Figure 11. EMSA supershift assays analyzing transcription factor binding to mCOX-2 promoter elements. A and B. Transcription factor binding to CRE-2. EMSA supershift assays were performed as described in the Methods section. The double-stranded ^{32}P-labeled CRE-2 probe was incubated with a nuclear extract from RAW 264.7 cells stimulated with LPS (200 ng/ml) for 0, 1, or 12 hr and either an IgG control antibody or an antibody to CREB or ATF-2 (A) or to phosphorylated c-Jun or ATF-1 (B). CREB, ATF-2, and P-c-Jun supershifted complexes are denoted with open arrows and the CRE-2 complex is indicated with the closed arrows. C. p65 and p50 binding to NF-κB probe (24). The double-stranded ^{32}P-labeled NF-κB probe was incubated with a nuclear extract from RAW 264.7 cells stimulated with LPS (200 ng/ml) for 0, 1 or 12 hr and either an IgG control antibody or an antibody to p50 or p65. Complexes of the NF-κB probe and p50/p65 heterodimers or p50/p50 homodimers are indicated with closed arrows. The supershifted (SS) complexes bound to antibody to p50 or p65 are indicated with open arrows.


A
Probe
CRE2 NF-kB
Cold probe NONE mCRE-2 mNF-kB double mut. WT
LPS (hr) 0 1 12 0 1 12 0 1 12 0 1 12 0 1 12
p65/p50
p50/p50
Free probes
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


B
CRE2 NF-kB
CRE2 NF-kB
CRE2 NF-kB
CRE2 NF-kB
Probe WT(57bp) mCRE2 mNF-kB double mut.
LPS (hr) 0 1 12 0 1 12 0 1 12 0 1 12
p65/p50
p50/p50
Free probes
1 2 3 4 5 6 7 8 9 10 11 12

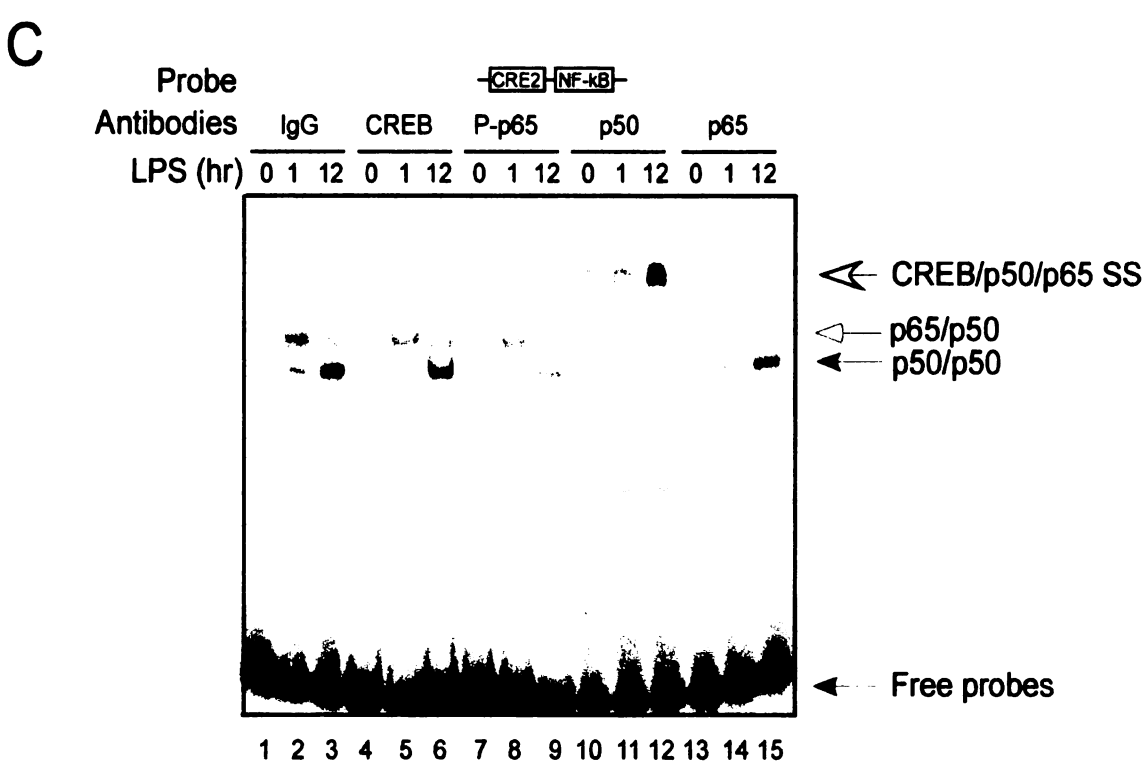


Figure 12. CREB and p65/p50 binding to a probe containing both the CRE-2 and the NF-κB site. An EMSA was performed using a double-stranded ^{32}P-labeled 57 bp probe containing the CRE-2 and NF-κB site and nuclear extracts from RAW 264.7 cells that has been stimulated with LPS (200 ng/ml) for 0, 1, or 12 hr. (A). The double stranded ^{32}P-labeled 57 bp probe was incubated with each of the three nuclear extracts in the presence and absence of a 100 fold molar excess of unlabeled probes containing mutations of either or both of the CRE-2 and the NF-κB site. (B) Double stranded ^{32}P-labeled 57 bp probes containing mutations of either or both of the CRE-2 and NF-κB was incubated with nuclear extracts from each time point. (C) Normal IgG or antibodies to CREB, phosphorylated p65, p65, or p50 was incubated with 59 bp probe and nuclear extracts. The CREB and p50 supershifted complexes are indicated with open arrows (C), p65/p50 complex is denoted with small open arrows, and p50 homodimers are indicated with closed arrows.

well as by CREB/ATF family members, antibodies to phosphorylted c-Jun, CREB, phosphorylated CREB, ATF-1, and ATF-2 were used in addition to antibodies to p65 and p50 for NF-κB element (Fig. 13). Consistent with previous EMSA anaysis, p65 binding was inducible and peaked at 1 hr and decreased after 4 and 12 hr of LPS stimulation whereas p50 binding increased with time. Phosphorylated CREB binding was detected even without LPS treatment, decreased after 1 hr but increased again after 12 hr of LPS stimulation. ATF-1 and ATF-2 are also bound constitutively to CRE-2 site (Fig. 13). It has been shown that phosphorylated CREB or phosphorylated p65 can recruit co-activators such as CBP/p300 to transcriptional response elements. For this reason, a CBP/p300 immunoblot was also performed to determine if CBP/p300 was present; however, little or no co-activator could be precipitated (Fig. 13).

Gel shift analysis revealed that the binding of nuclear proteins to the AP-1 site (5'-ACAGAGTCACCAC-3'; Table III) is induced by LPS stimulation but that the extent of binding then remains about the same 1 and 12 hr after initiating LPS treatment (Fig. 14). To identify the transcription factors bound to the AP-1 site, supershift assays were performed using antibodies reactive with c-Jun, phosphorylated c-Jun, c-fos, Jun D, Fra 1, Fra 2, ATF-1, ATF-2, and CREB. This analysis indicated that the AP-1 site is bound primarily by phosphorylated c-Jun and c-fos (Fig. 14A). No binding of members of the other AP-1 or CREB/ATF transcription factor family to the AP-1 site was observed (Fig. 14B, data not shown). Specific binding activity of phosphorylated c-Jun was almost undetectable in untreated control cells but was increased considerably after stimulation of macrophages with LPS. Binding of c-fos to the AP-1 site was increased at 1 hr treatment of LPS but absent at 12 hr of LPS treatment, suggesting that the c-fos is specifically

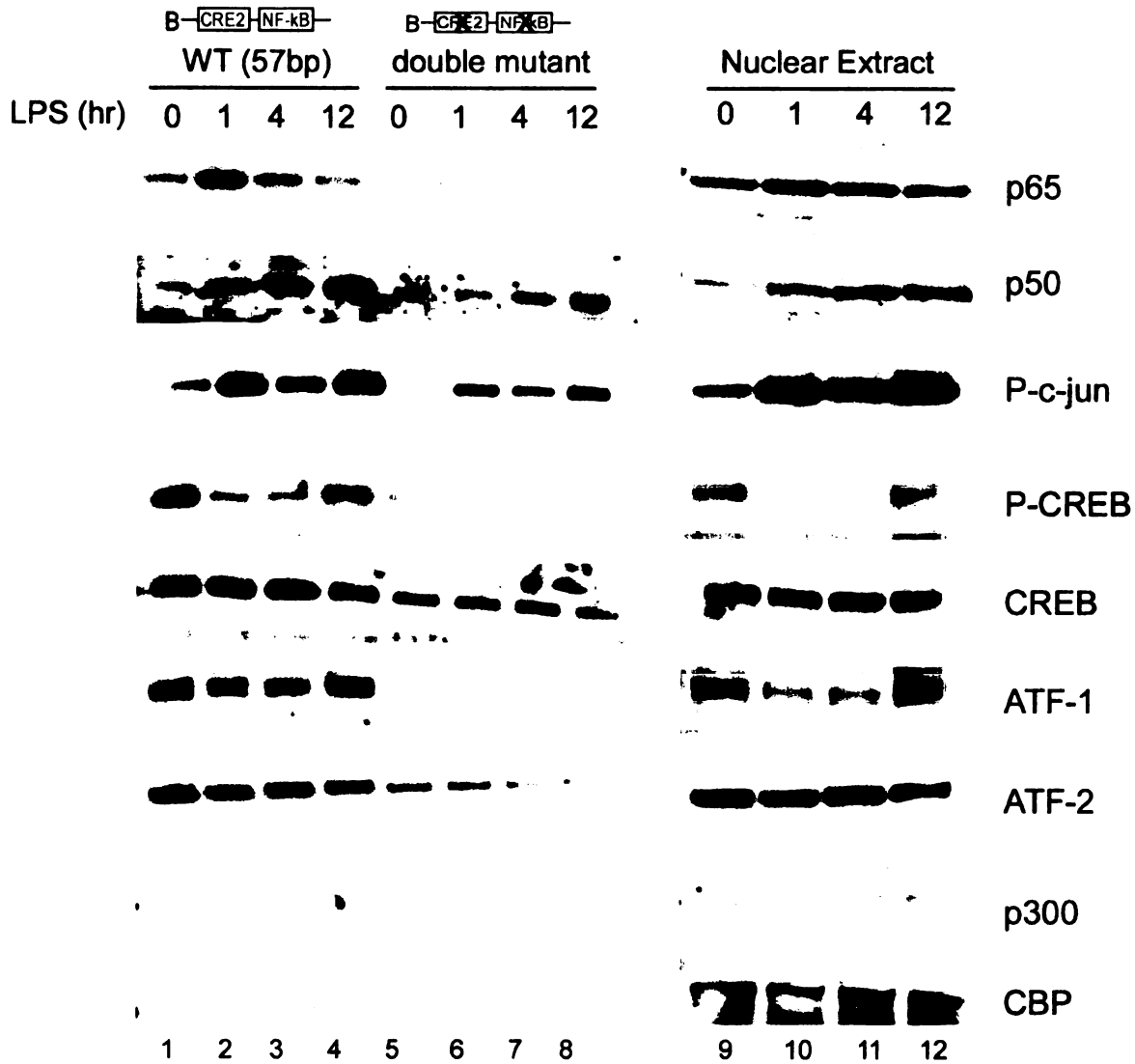


Figure 13. Representative immunoblots showing transcription factor binding to the biotinylated probe that contains the CRE-2 and the NF-κB site in the presence or absence of LPS stimulation. A double stranded biotin-labeled 57 bp probe containing the CRE-2 and the NF-κB site was prepared and a probe with mutations of both elements as a negative control. Nuclear extracts from RAW 264.7 cells treated with LPS at 1, 4, or 12 hr (200 μg) were incubated with the probe (2 μg) then streptavidin-beads was added to precipitate the protein-DNA complexes. Bound protein was eluted and subjected to western blottings with anti-p65, -p50, -P-c-Jun, -P-CREB, -CREB, -ATF-1, -ATF-2, -p300, or -CBP antibodies as shown in the left hand panel. 20 μg of nuclear extract from different time points were subjected to western blotting as shown in the right hand panel.

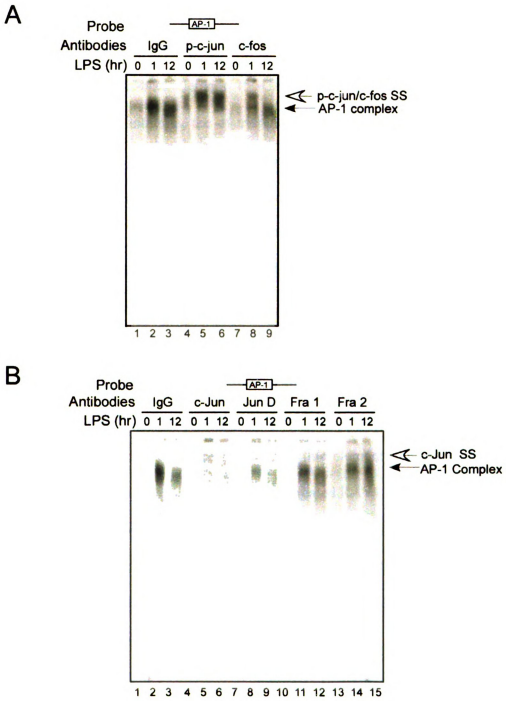


Figure 14. AP-1 transcription factor binding to a COX-2 AP-1 probe.
A and B. EMSA supershift assays were performed using a double-stranded ^{32}P-labeled AP-1 probe. The probe was incubated with nuclear extracts from RAW 264.7 cells stimulated with LPS (200 ng/ml) for 0, 1, or 12 hr and either an IgG control antibody or an antibody to (A) phosphorylated c-Jun or c-fos, (B) c-Jun, Jun D, Fra 1, or Fra 2. The AP-1 complex is indicated with closed arrows and the (A) P-c-Jun and c-fos, (B) c-Jun supershifted complex is indicated with open arrows.

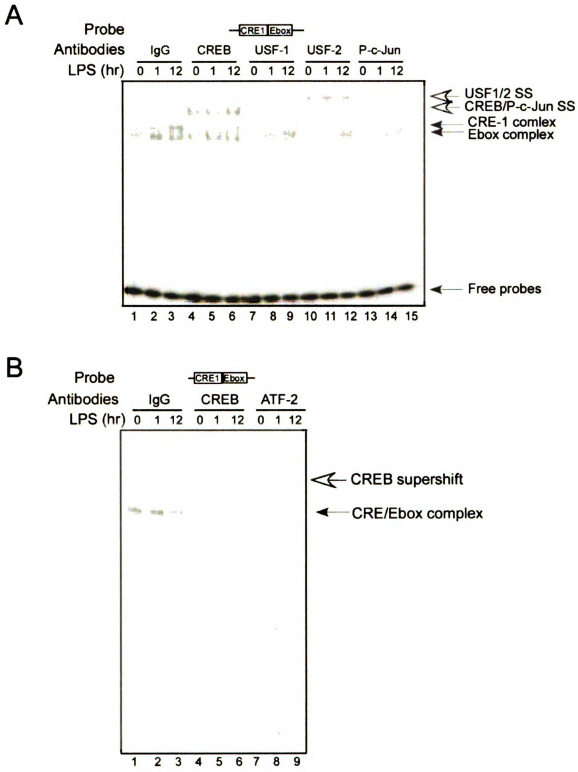
playing an important role in the early phase of COX-2 induction.

To identify nuclear proteins that bind to the overlapping CRE-1 (5'-ACGTCA-3'; Table III) and E-box element (5'-CACGTG-3'; Table III), a ^{32}P-labeled 25 bp oligonucleotide containing the overlapping CRE-1 and E-box was prepared for EMSA supershift assays (Fig. 15). As was observed at the NF-κB probe, two distinct complexes were formed with the 25 bp CRE-1/E-box probe (Fig. 15A, lanes 1-3). The lower mobility complex was shifted by anti-CREB antibody (Fig. 15A, lanes 4-6 and Fig. 15B, lanes 4-6) and the higher mobility complex was shifted by USF-1 (Fig. 15A, lane 7-9) or USF-2 antibodies (Fig. 15A, lane 10-12). Additionally, low amount of phorphorylated c-Jun was inducibly bound to CRE-1/E-box probe. However, ATF-2 antibody failed to shift the complex, suggesting the absence of ATF-2 in the CRE-1/E-box complex. Super shifting with CREB, USF-1, and USF-2 antibodies could shift whole the complex (Fig. 15C). Transcription factor recruitment to this element appears to be constitutive, as similar levels of binding are observed in untreated RAW 264.7 cells and after 1 and 12 hr of LPS stimulation. Although mutation of the E-box lead to a significant increase in COX-2 transcription, overexpression of USF-1 or USF-2 failed to repress COX-2 promoter activity (Chapter 3) suggesting that the repression does not directly involve USF-1 or USF-2 binding to the E-box.

Since AP-1 and CRE-1 sites on COX-2 promoter are only 2 bp distant away from each other, it is possible that they could compete for binding to nuclear proteins. To test this, a ^{32}P-labeled 39 bp oligonucleotide containing both AP-1 and CRE-1 site was prepared for EMSA and supershift assays. As shown in Fig. 16A, three complexes were formed with the 39 bp probe (Fig. 16A, lane 1-3); two lower complexes were formed

when mAP-1 probe was used (Fig. 16A, lane 4-6), upper complex was formed when mCRE-1 probe was used (Fig. 16A, lane 7-9), indicating that the AP-1 complex migrated slower than CRE-1/E-box complex. AP-1 and CRE-1 complexes were abolished using probe containing mutations on both sites (Fig. 16A, lane 10-12). A similar experiment was performed using cold probe as a competitor (Fig. 16B). Phosphorylated c-Jun, CREB, USF-1, and USF-2 antibodies were used in supershift experiments to identify the protein composition of the complexes formed with the AP1/CRE-1/E-box probe (Fig. 17). The AP-1 complex was supershifted by phosphorylated c-Jun antibody (Fig. 17, lane 4-6), CRE-1 complex was supershifted by CREB antibody (Fig. 17, lane 7-9), and E-box complex was supershifted USF-1 antibody (lane 10-12), or USF-2 antibody (lane 13-15). The results indicate that phosphorylated c-Jun, CREB, and USF-1/-2 binding was mutually exclusive and the binding pattern did not change when a longer probe was used.

***In vivo* association of transcription factors with the mCOX-2 promoter in LPS-stimulated RAW 264.7 cells.** Results of the EMSA supershift assays suggest that CREB, p65, and p50, phosphorylated c-Jun and c-fos and USF-1/-2 transcription factors can bind to the CRE-1 and CRE-2, NF-κB, AP-1, and the E-box, respectively. Chromatin immunoprecipitation (ChIP) analysis were performed to determine the composition of *trans*-acting factors associated with the mCOX-2 promoter in intact RAW 264.7 cells after 0, 1 or 12 hr of LPS stimulation (Fig. 18). In agreement with the EMSA supershift results, ChIP assays revealed that CREB and USF-1/-2 are constitutively associated with the COX-2 promoter (Fig. 18C, 18F). As predicted by the observed changes in the


A
Probe
CRE1 Ebox
Antibodies
IgG
CREB
USF-1
USF-2
P-c-Jun
LPS (hr)
0 1 12 0 1 12 0 1 12 0 1 12 0 1 12
USF1/2 SS
CREB/P-c-Jun SS
CRE-1 comlex
Ebox complex
Free probes
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
Probe
CRE1 Ebox
Antibodies
IgG
CREB
ATF-2
LPS (hr)
0 1 12 0 1 12 0 1 12
CREB supershift
CRE/Ebox complex
1 2 3 4 5 6 7 8 9

C

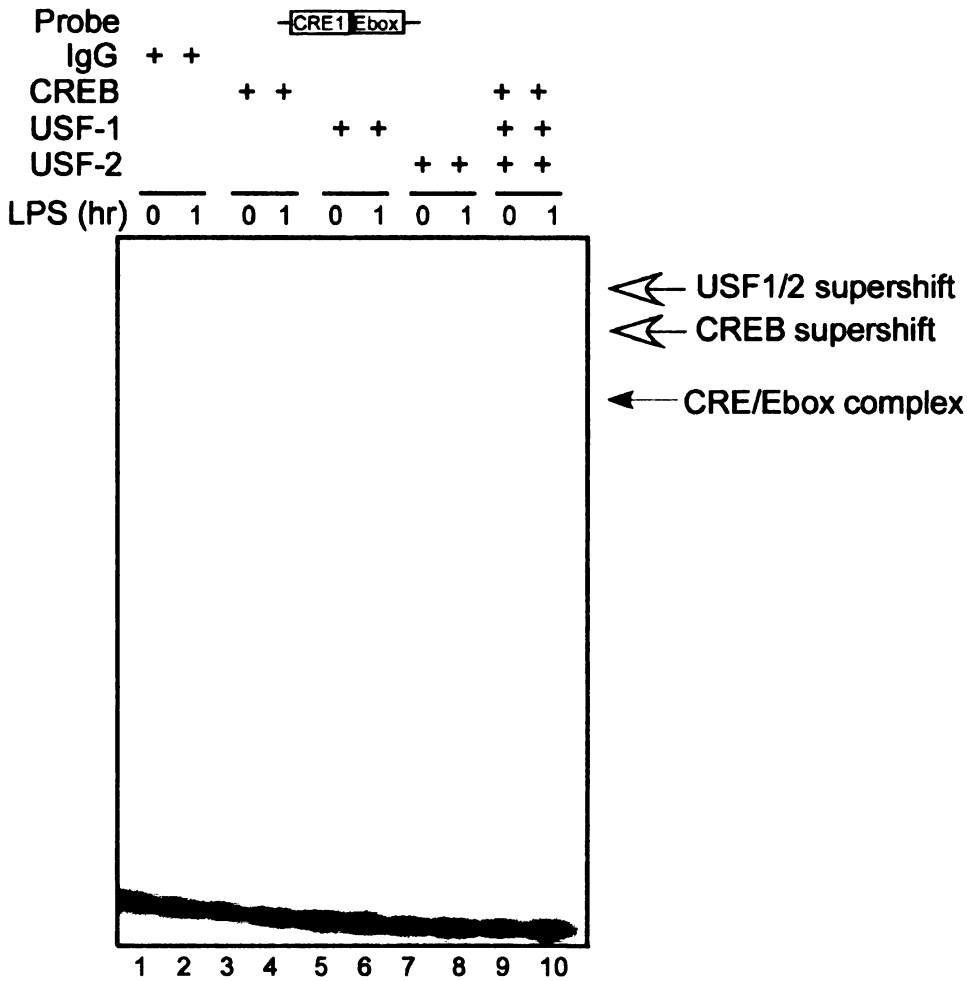


Figure 15. CREB, USF-1, USF-2, and phosphorylated c-Jun binding to a COX-2 probe with the overlapping CRE-1/E-box probe. EMSA supershift assays were performed with a double-stranded ^{32}P-labeled COX-2 probe containing the overlapping CRE/E-box. The probe was incubated with a nuclear extract from RAW 264.7 cells stimulated with LPS (200 ng/ml) for 0, 1, or 12 hr (0 or 1 hr for C) and either an IgG control antibody or an antibody to (A and C) CREB, USF-1, USF-2, or phosphorylated c-Jun, (B) CREB or ATF-2. The CRE-1, and E-box complexes are indicated with closed arrows and the supershifted complexes are indicated with open arrows.

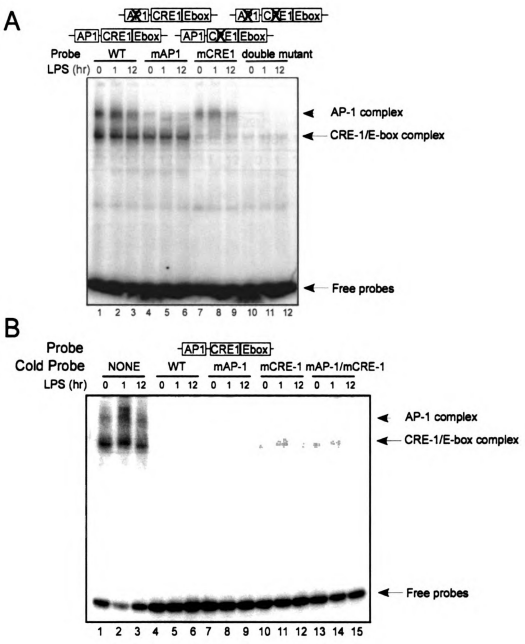


Figure 16. Specific binding of nuclear protein to the AP-1/CRE-1/E-box probe. A double-stranded ^{32}P-labeled 39 bp probe containing AP-1 and CRE-1/E-box site was incubated with nuclear extracts from RAW 264.7 cells stimulated without or with LPS (200 ng/ml) for 1 or 12 hr. A. WT 39 bp probe that did not contain any mutations or probes with a mutation on either or both AP-1 and CRE-1 were used for EMSA. AP-1 and CRE-1/E-box complexes are indicated with the arrows. B. WT ^{32}P-labeled 39 bp probe was incubated with each of the three extracts in the presence or absence of 20 molar excess of unlabeled WT, mutant AP-1, mutant CRE-1, or mutant AP-1/mutant CRE-1. AP-1 and CRE-1/E-box complexes are indicated with the arrows.

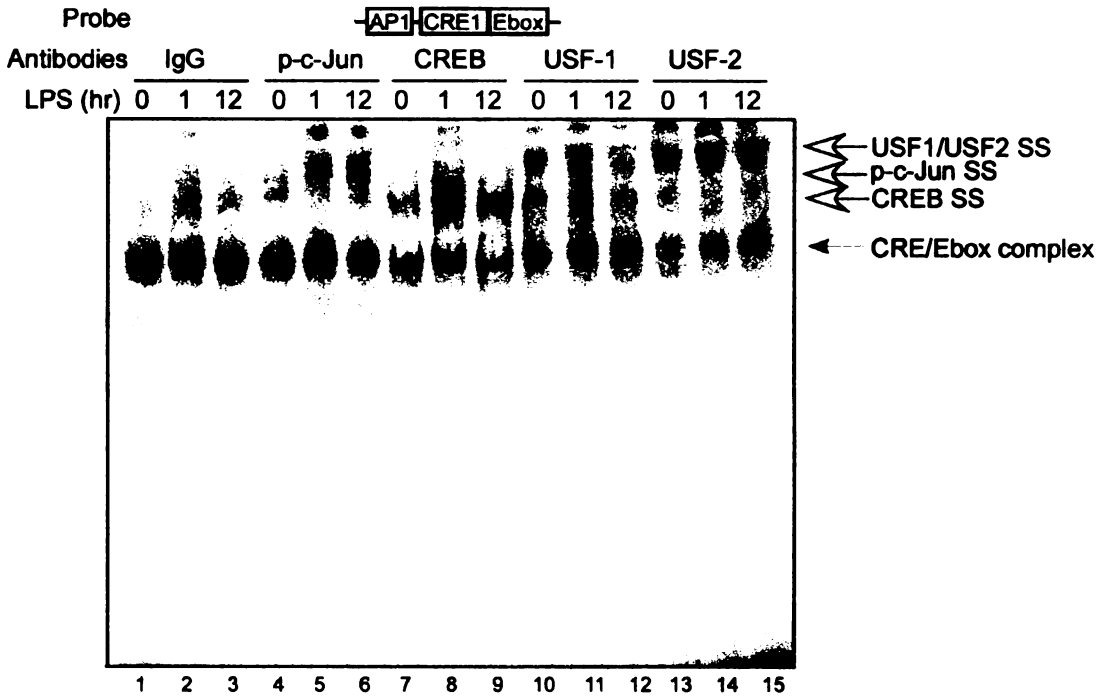


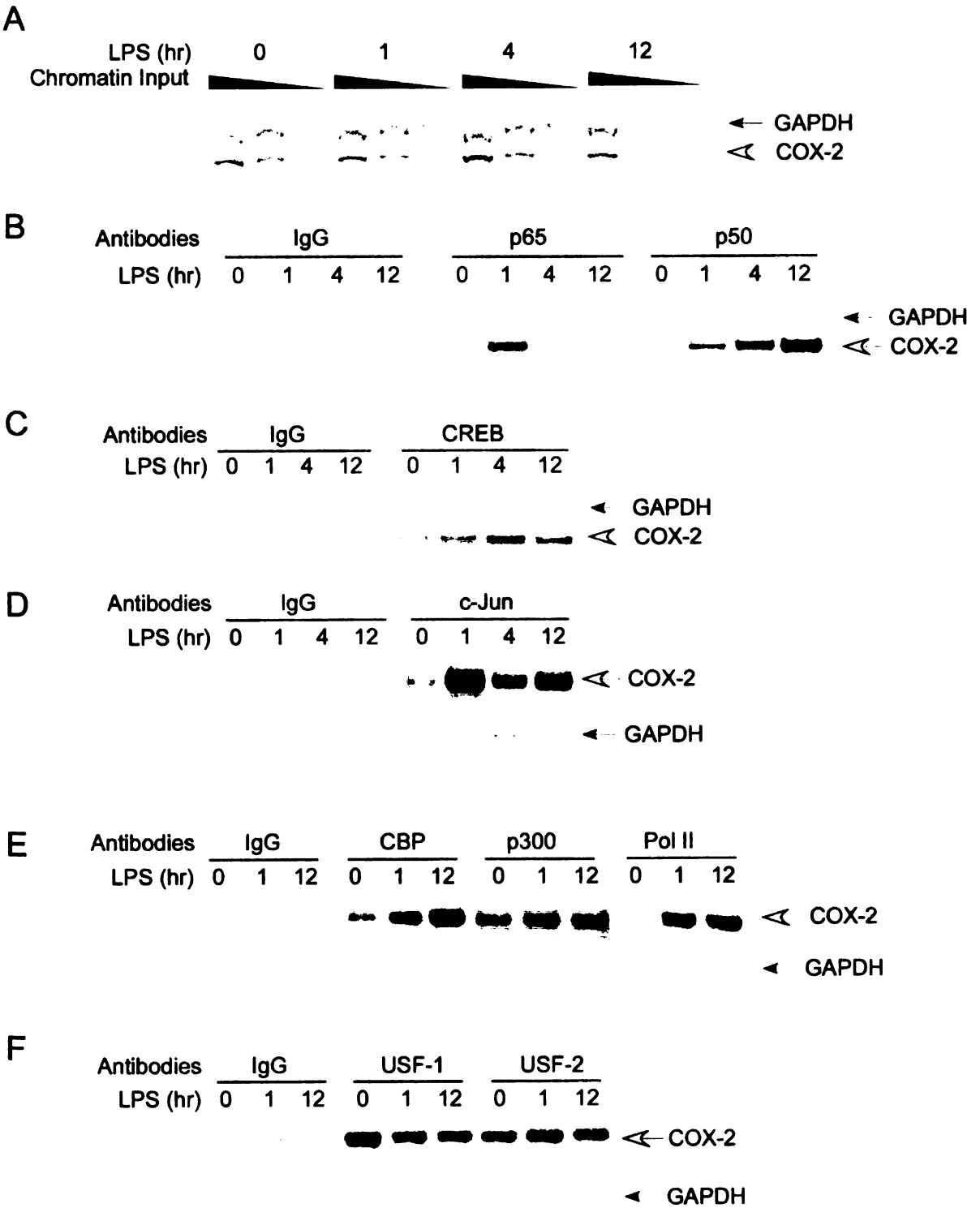
Figure 17. CREB/ATF and AP-1 transcription factors bind to the AP-1/CRE-1/E-box probe. An EMSA supershift assay was performed. A double-stranded ^{32}P-labeled 41 bp probe containing AP-1 and overlapping CRE-1/E-box element was incubated with a nuclear extract from RAW 264.7 cells stimulated with LPS (200 ng/ml) for 0, 1, or 12 hr, and either a control IgG antibody or an antibody to phosphorylated c-Jun, CREB, USF-1, or USF-2. The P-c-Jun, CREB, USF-1, and USF-2 supershifted complexes are indicated with open arrows and the CRE-1/E-box complexes are indicated with closed arrows.

composition of the NF-κB dimer by EMSA supershifts, p65 binding is increased at 1 hr and decreased after 4 hr whereas p50 association increases from 0-12 hr (Fig. 18B). Similar to the *in vitro* pattern of c-Jun recruitment, c-Jun binding *in vivo* is increased 1 hr after LPS treatment, is decreased at 4 hr and then is increased again at 12 hr (Fig. 18D). Consistent with increased COX-2 gene transcription (Fig. 9), the transcriptional coactivators CBP and p300 as well as RNA polymerase II associate with the COX-2 promoter in response to LPS treatment (Fig. 18E).

Collectively, the results the EMSA supershift assays and the ChIP analyses are qualitatively and semi-quantitatively consistent and suggest that there is a distinct temporal pattern to the binding of different transcription factors to different *cis*-acting elements in the COX-2 promoter in response to LPS stimulation.

Effect of dominant negative transcription factors on COX-2 transcriptional activation. To investigate the mechanism whereby CREB regulates COX-2 transcription, a dominant negative CREB called ACREB, which cannot bind DNA, was cotransfected into RAW264.7 cells along with the COX-2 promoter luciferase reporter construct (Fig. 19A). Transfection of increasing amounts of ACREB lead to a reduction in LPS-inducible luciferase activity (Fig. 19A), suggesting that recruitment of endogenous cofactors to the promoter by native CREB is required for LPS-induced COX-2 transcription in RAW 264.7 cells. The COX-2 promoter has two CRE sites, both of which can bind CREB (Fig. 11, 15). To determine the impact of the interaction with CREB at each of the CRE sites, the ACREB expression vector was co-transfected with reporter constructs driven by either the native mCOX-2 promoter or a version of the

Figure 18. Chromatin immunoprecipitation (ChIP) assays to identify transcription factors associated with the mCOX-2 promoter in LPS-stimulated RAW 264.7 cells. A. RAW 264.7 cells were stimulated with LPS for various times (0, 1, 4 or 12 hr) and PCR analyses were performed on three-fold serial dilutions of the input chromatin to establish that the amounts of PCR reaction products are proportional to the amounts of starting material. B-F. PCR analysis of DNA from the ChIP assays. Mock immunoprecipitation with IgG (B-F) was performed as a negative control and PCR was performed using the same primers. Chromatin from the RAW 264.7 cells was diluted 1:10 and the samples were processed through the ChIP protocol as described in the Methods section. Antibodies to p65 or p50 were used for (B), antibody to CREB-1 was used for (C), c-Jun antibody was used for (D), antibodies to CBP, p300, RNA polymerase II were used for (E), and USF-1 and USF-2 for (F). GAPDH primers were used as negative controls for the PCR.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1
4
12
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COX-2
B
Antibodies
IgG
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0 1 4 12
0 1 4 12
0 1 4 12
GAPDH
COX-2
C
Antibodies
IgG
CREB
LPS (hr)
0 1 4 12
0 1 4 12
GAPDH
COX-2
D
Antibodies
IgG
c-Jun
LPS (hr)
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0 1 4 12
COX-2
GAPDH
E
Antibodies
IgG
CBP
p300
Pol II
LPS (hr)
0 1 12
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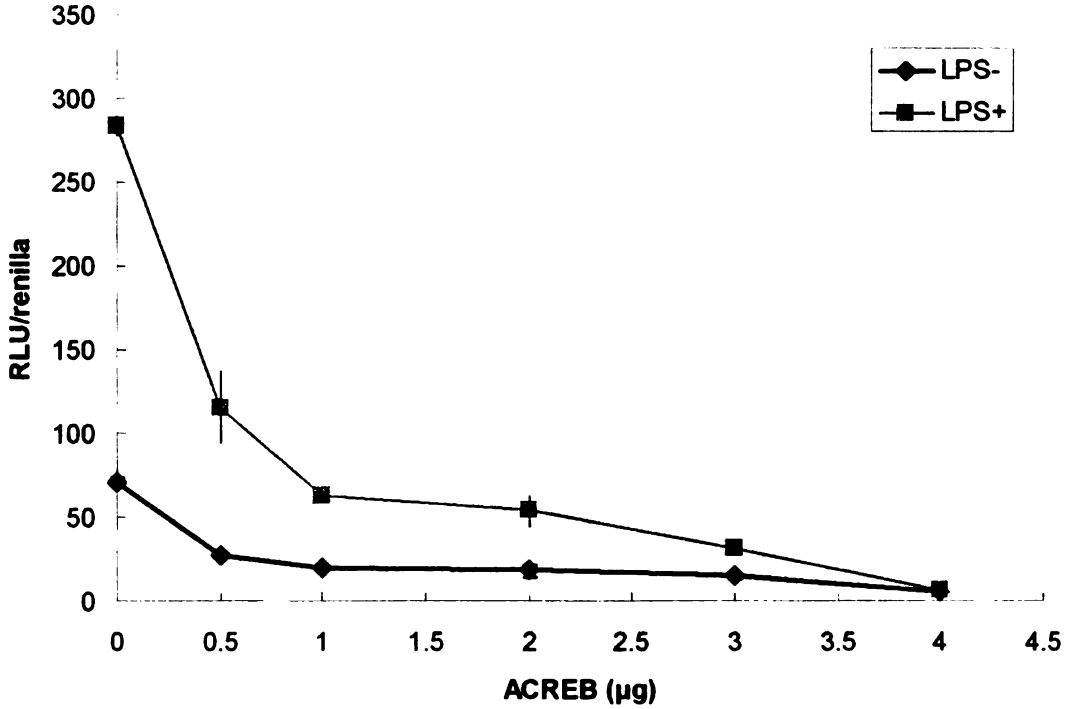
0 1 12
COX-2
GAPDH
F
Antibodies
IgG
USF-1
USF-2
LPS (hr)
0 1 12
0 1 12
0 1 12
COX-2
GAPDH

promoter with individual mutantions in either CRE-1 or CRE-2 (Fig. 19B). Although expression of the mutant CRE-1 reporter construct lead to a more substantial reduction in reporter activity than expression of the mutant CRE-2 construct, both mutations resulted in a significant reduction in reporter activity compared to intact mCOX-2 promoter. Furthermore, cotransfection of each of the mutant CRE reporters with ACREB resulted in a comparable fold reduction in reporter activity (Fig. 19B). These results indicate that LPS-induced expression of mCOX-2 depends on CREB binding through both the CRE-1 and the CRE-2 and suggests that another transcription factor may also be activating COX-2 expression through the CRE-1.

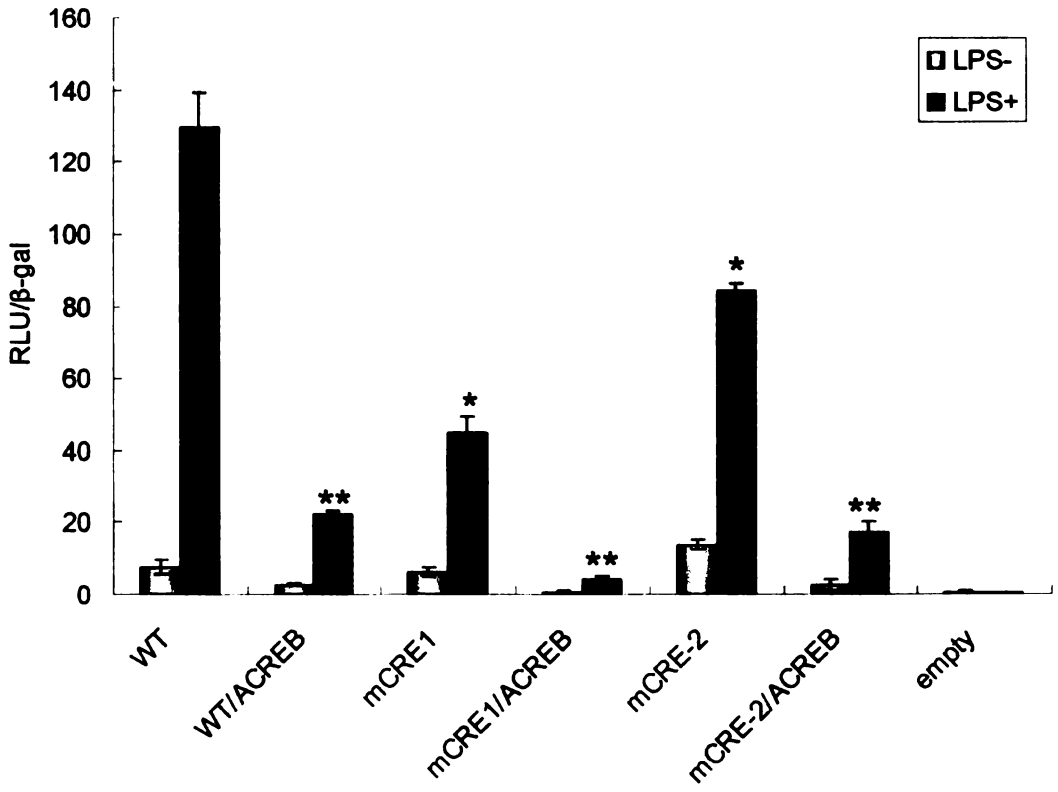
To evaluate the role of NF-κB activation in this system, RAW 264.7 macrophages were cotransfected with the COX-2 reporter construct and a dominant negative IκB (IκB S/R) that fails to release p65/p50 after activation and thus traps NF-κB in the cytoplasm (Fig. 19C). Again, transfection of increasing amounts of the dominant negative expression vector lead to a reduction in mCOX-2 transcriptional activity. This suggests that p65/p50 is also required for COX-2 transcription.

CBP and p300 are both involved in COX-2 transcriptional regulation. CBP and p300 are homologous coactivators that interact with many trans-acting transcription factors including CREB, NF-κB, and c-Jun (31, 32). To determine the influence of CBP and p300 on LPS-induced COX-2 transcriptional regulation, we overexpressed each of these coactivators and evaluated the effect on COX-2 reporter activity. In both cases, LPS-inducible COX-2 luciferase activity was increased with increasing amounts of CBP and p300 expression vector (Fig, 19D, 19E). This suggests that transcriptional

A


350
300
250
200
150
100
50
0
RLU/renilla
LPS-
LPS+
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
ACREB (µg)

B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120
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60
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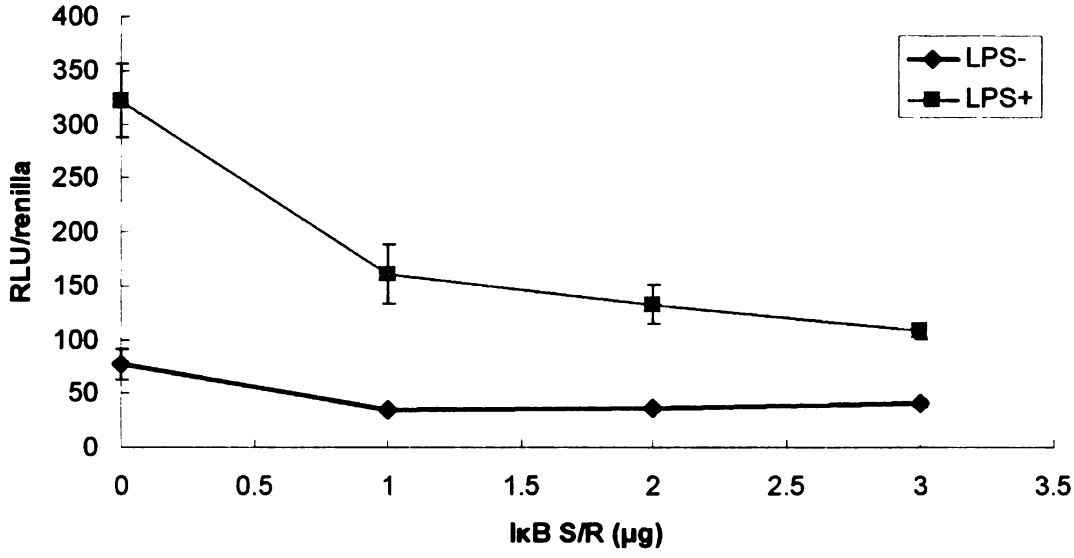
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350
300
250
200
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50
0
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LPS+
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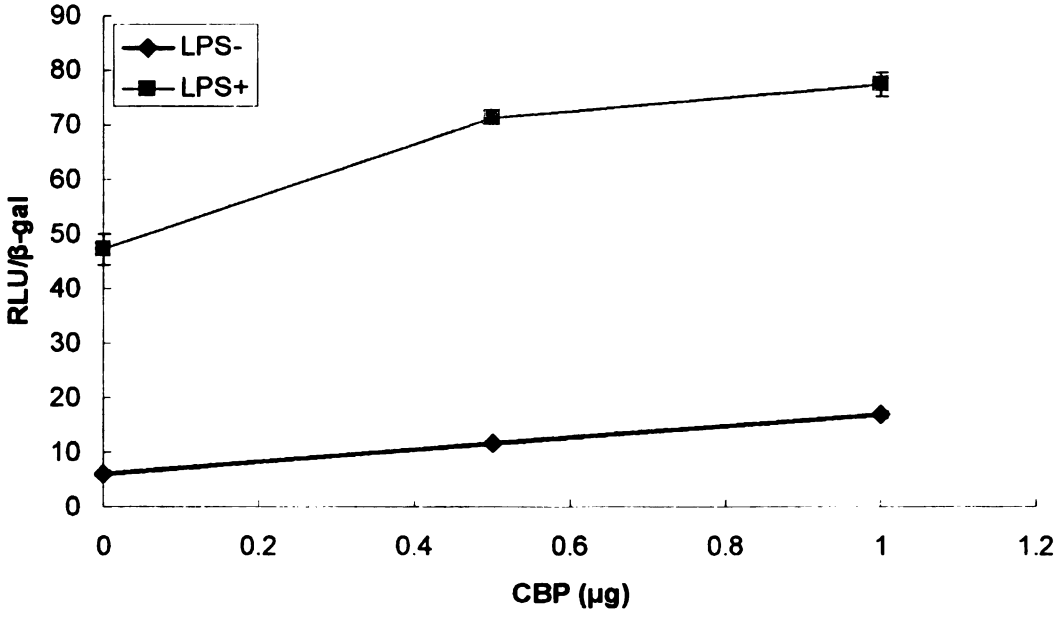
0.5
1
1.5
2
2.5
3
3.5
IκB S/R (μg)

D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30
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0.6
0.8
1
1.2
CBP (μg)

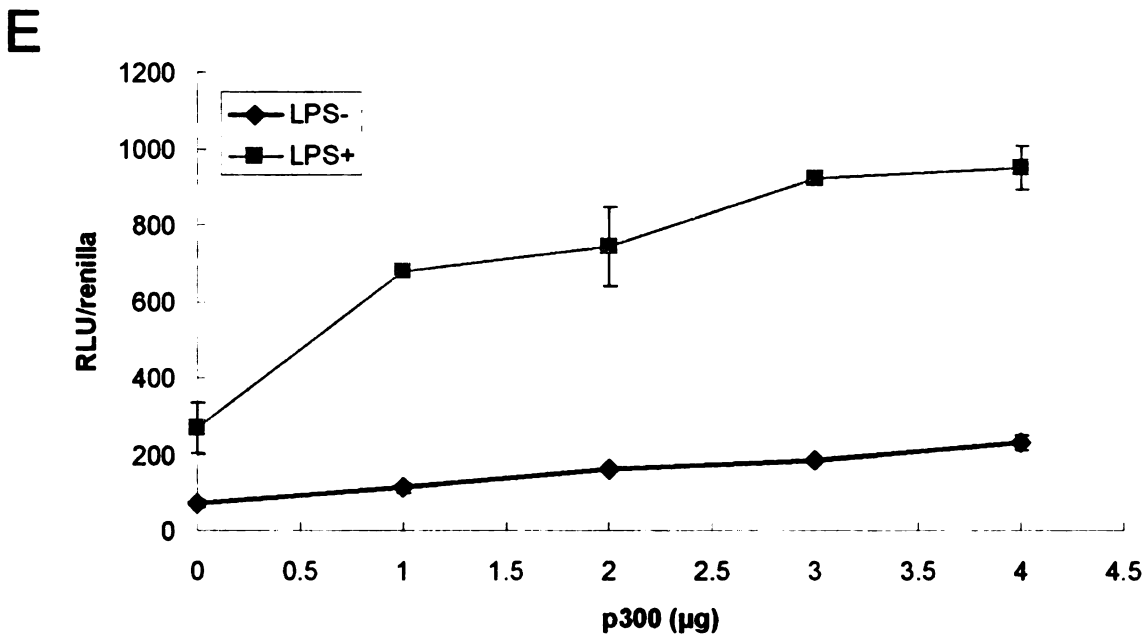


Figure 19. Functional assays of COX-2 transcriptional regulation in LPS-treated RAW 264.7 cells. RAW 264.7 cells were transiently co-transfected with the indicated amounts of ACREB expression plasmid (A), IκB expression plasmid (C), CBP expression plasmid (D), or p300 expression plasmid (E) as well as 1 μg of COX-2 luciferase promoter reporter plasmid and 0.5 μg of the renilla (A, C, and E) or β-galactosidase expression plasmid (D). ACREB (cannot bind to DNA) and IκB S/R (traps p65/p50 heterodimer in the cell cytoplasm) suppressed COX-2 promoter activity (A and C respectively) and CBP and p300 overexpression increased COX-2 promoter activity (D and E) in LPS-treated RAW 264.7 cells. Cells were stimulated with 200 ng/ml of LPS for 12 hr after 24 hr of transfection and cell extracts were prepared and processed to measure luciferase activity. Data represent two independent experiments performed in duplicate. B. Both CRE-1 and CRE-2 element require CREB binding for LPS-stimulated COX-2 transcription in RAW 264.7 cells. RAW 264.7 cells were transiently co-transfected with 1 μg of ACREB expression vector and 1 μg of wild type (WT), mutant CRE-1, or mutant CRE-2 COX-2 promoter construct and 0.5 μg of the β-galactosidase expression plasmid for measuring transfection efficiency. Data represent three independent experiments performed in duplicate. Error bars represent the standard deviation of the average values attained from all three experiments. *P<0.05 and **P<0.01 for WT plasmid-transfected LPS-treated cells vs. mutant COX-2 construct and/or ACREB expression plasmid-transfected LPS-treated cells.

coactivators, CBP and p300 are involved in LPS-induced COX-2 transcriptional activation in RAW 264.7 cells.

P50 and phosphorylated c-Jun are uniquely required for the middle phase of COX-2 gene transcription. Consistent with Northern blot analysis (Fig. 9), semi-quantitative RT-PCR analysis showed that COX-2 mRNA levels increased over the 12 hr period of LPS treatment and that there are three phases (Fig. 20, top panel). Our ChIP data suggest that binding of both p50 homodimers and phosphorylated c-Jun to the COX-2 promoter increase from 4 to 12 hr of LPS treatment (Fig. 18B and 18D). To determine the relative contribution of phosphorylated c-Jun and p50 at the middle and late phases of COX-2 induction, JNK and p50 inhibitors were added separately and in combination 3.5 hrs after initiating the LPS treatment and the COX-2 mRNA levels were analyzed by semi-quantitative RT-PCR at 4, 6, 9 and 12 hr (Fig. 20). COX-2 gene transcription was reduced by about half by the individual inhibitors and was completely abrogated at 6 hrs after LPS treatment in cells treated with both JNK and p50 inhibitors. However, COX-2 expression begins to recover at the 9 and 12 hr time points (Fig. 20) likely as a result of the involvement of other transcription factors such as phosphorylated CREB or C/EBP. This experiment demonstrates that the contribution of different transcription factors varies with time during LPS-induced COX-2 expression.

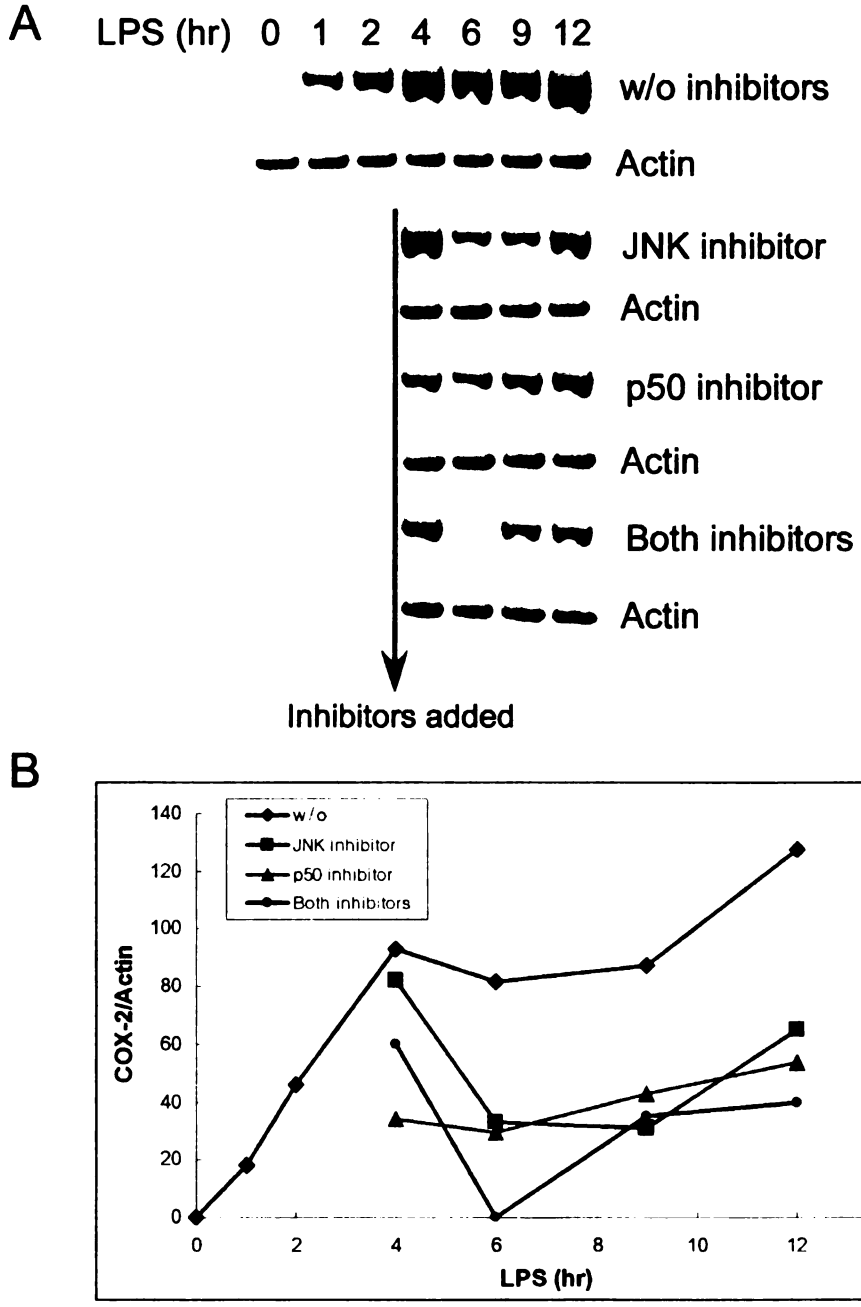


Figure 20. Effects of JNK and p50 inhibitors on COX-2 mRNA induction in LPS-treated RAW 264.7 macrophages as determined by semi-quantitative RT-PCR. A. Cells were stimulated for 1, 2, 4, 6, 9, or 12 hr with or without LPS (200 ng/ml), and JNK or p50 inhibitors were added after 3.5 hrs after LPS treatment as described in the Methods section. Total RNA was extracted and subjected to RT-PCR using specific primers for COX-2 and β-actin (internal control), and agarose gels stained with cybergreen. B. Densitometric analysis combining results from two independent experiments. COX-2 values were normalized to β-actin values in all experiments.

Discussion

LPS and certain cytokines and growth factors increase the expression of COX-2 which, in turn, leads to the formation of pro-inflammatory prostaglandins. Specific COX-2 inhibitors such as celecoxib and rofecoxib are effective anti-inflammatory and analgesic agents. While the mechanism of COX-2 transcription in macrophage cells is complex, resolving the complicated network of regulatory events controlling COX-2 expression during inflammation will likely uncover new potential clinical targets for lowering COX-2 activity in a cell specific manner. In this study, we have used LPS-treated RAW 264.7 cells as a model to study the role of COX-2 transcription and how it is controlled during an acute inflammatory response. We have (a) identified three previously uncharacterized, functional *cis*-elements in the COX-2 promoter in addition to the three that had been reported previously, (b) catalogued the binding of transcription factors to each element over a 12 hr time course following LPS treatment, (c) showed that there are at least three distinct temporal phases of COX-2 transcriptional activation, and (d) provided evidence that each phase involves the participation of unique combinations of *cis*-elements and *trans*-activating factors.

LPS treatment of RAW264.7 cells increases the rate of COX-2 transcription. We first established using nuclear run-on experiments and measurements of mRNA accumulation that LPS treatment causes an increase in the rate of COX-2 gene transcription that continues for 12 hr but occurs in distinct early, middle and late phases. COX-2 transcription is rapid for the first 4 hrs of LPS treatment (early phase), then there is a slowing of the transcription rate during the next 2-4 hrs (middle phase), and finally,

the rate of COX-2 gene transcription increases again between 8-12 hrs (late phase). The sustained COX-2 mRNA expression observed in LPS-treated RAW 264.7 cells contrasts with the more transient inducible COX-2 expression observed in other cell types and in response to other agonists (e.g. serum-induced COX-2 expression in murine fibroblasts (17, 33) where gene transcription rates also increase rapidly but with COX-2 mRNA levels peaking within one hr and then decreasing to basal levels within 2-3 hr. Although the rate of COX-2 transcription is significantly increased in LPS-treated RAW264.7 cells, it has been previously reported that the increased amount of COX-2 mRNA in response to LPS is a result of a decrease in the rates of post-transcriptional processing (34). Our studies focus on the transcriptionally modulated aspects of COX-2 expression and cannot rule out the participation of mRNA stability in this process.

***Cis*-acting elements functional in LPS-induced COX-2 gene expression.** Previously published reports have identified several *cis*-acting elements of the murine COX-2 promoter that are necessary for a response to LPS. These elements include a CRE (CRE-1) at -59/-52 (7), a C/EBP site at –138/-130 (28), and an NF-κB response element at -401/-393 (5, 6, 9, 35) (Fig. 8). In addition, it has been shown that inhibition of NF-κB activation with specific inhibitors or decoy oligonucleotides inhibits COX-2 activation (36-40). Using promoter reporter assays, we have identified a second CRE (CRE-2) located at -447/-440 and established that the AP-1 site located at -73/-61 is necessary for maximal LPS induction of the murine COX-2 gene (Fig. 8). Additionally, we have provided evidence for a role of the E-box element at -53/-48 in repression of mCOX-2 transcription.

***Trans*-acting factors functional in LPS-induced COX-2 gene expression.** Using EMSA and ChIP assays, we found that AP-1 or CREB bind to the CRE-1 site. Studies with dominant negative ACREB suggest that CREB binding to both CRE-1 and CRE-2 is important in COX-2 transcriptional activation. USF transcription factors bind to the E-box that overlaps the CRE-1 site. However, binding of AP-1 or CREB to the CRE-1 site and binding of USF-1/2 to the E-box are mutually exclusive. Unlike other systems where the E-box is involved in activation of COX-2 transcription (15, 41, 42), our promoter reporter assays indicate that the E-box in the murine COX-2 promoter is involved in repression in RAW 264.7 cells. Mestre *et al.* (14) reported that the E-box mediates COX-2 transcriptional activation in response to LPS and involves USF-1 binding to the E-box; however, their study used a reporter gene under the control of a short human COX-2 promoter construct (-327/+59) that did not contain the CRE-2 or NF-κB element. There could also be species differences in COX-2 transcriptional regulation that complicate the interpretation of the results. There are precedents for an E-box mediating repression in other cell types. In mesangial cells USF-1 and USF-2 trans-repress IL-1β-induced iNOS transcription by binding to the E-box element (43). However, in our studies USF-1 and USF-2 overexpression did not lower COX-2 promoter activity. This suggests that an as yet unidentified repressor complex binds to the E-box in LPS-treated RAW 264.7 cells to mediate transcriptional repression.

The AP-1 element has been shown to be required for COX-2 promoter activation in herpersvirus 6 (HHV-6)-infected monocytes (44), PMA-treated MC3T3 cells (45), and fluid shear stress-treated osteoblasts (46). However, the AP-1 site in LPS-induced COX-2 transcription in RAW 264.7 cells had not previously been studied. Our results establish

that mutation of the AP-1 site causes a decrease in promoter activity similar in magnitude to that caused by mutation of CRE-1. Additionally, as shown by EMSA supersift assays, members of the AP-1 transcription factor family--phosphorylated c-Jun and c-fos--bind to the AP-1 site and activate the COX-2 gene at early time points. Phosphorylated c-Jun homodimers are the predominant species bound to this site and play an important role in the middle and late phases of COX-2 transcription.

We have also discovered that the p50/p65 heterodimer and the p50 homodimer bind to the NF-κB site in a temporally regulated manner following LPS treatment. The p50/p65 dimer predominates in the early phase and the p50 homodimer predominates in the middle and late phases of LPS-induced COX-2 gene transcription.

CREB/ATF transcription factors bind to the CRE-2 site and studies with dominant negative CREB suggest that the regulatory action of CREB occurs through the CRE-2 site as well as the CRE-1 site.

Although not examined in the present studies, others have documented the binding of several C/EBP transcription factors the C/EBP response element of the COX-2 promoter (25, 47).

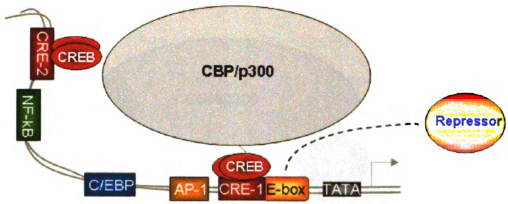
Putative c-Ets sites at -734/-725 and -329/-320 in the mCOX-2 promoter have been previously described as mediating COX-2 transcriptional activation in response to LPS induced phosphorylation of PU.1 (48). We were unable to identify a functional c-Ets element using promoter analyses. Sequence analysis of the mCOX-2 promoter using the Genomatrix program (www.genomatrix.de) located a potential c-Ets site (5'-GAGGAA-3') at -312/-306, but this site is different from those previously reported. The study that implicated PU.1 in mediation of COX-2 transcription in LPS-treated RAW

264.7 cells did not include promoter assays or evidence of PU.1 binding to an c-Ets site. If PU.1 is functional in this system, it is likely binding to an element other than an c-Ets site or activating COX-2 transcription indirectly.

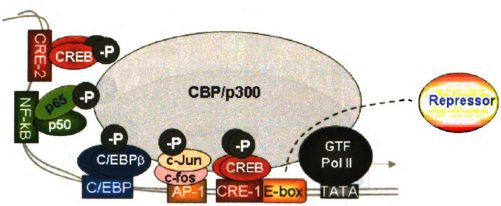
Dynamics of COX-2 transcriptional activation. The results of nuclear run-on assays showing that there are three different phases of transcriptional activation and EMSA and ChIP assays showing differences in the composition of the transcription factors bound to *cis*-elements in the COX-2 promoter at different times after LPS treatment suggest that the process of COX-2 gene activation involves sequential, coordinated events. In testing this concept, we found that when inhibitors of Jun kinase and p50 translocation were added together to RAW 264.7 cells 3.5 hr after initiating LPS treatment that transcription was completely blocked at 6 hr, but that during the period from 8-12, only partial inhibition of transcription was observed. Based on these observations, we developed a model illustrating events that are likely occurring at the COX-2 promoter at different times after LPS treatment (Fig. 21).

Even before LPS stimulation, CREB, USF-1 and USF-2 are bound to their cognate CRE-2, CRE-1 or E-box response elements within the mCOX-2 promoter (Fig. 21). In contrast, there is little or no binding of cognate transcription factors to the NF-κB, C/EBP or AP-1 sites. CBP, p300, and Pol II are associated with the COX-2 promoter at low levels. The presence of these activating components may permit basal COX-2 expression. We have not examined how these latter factors are recruited to the COX-2 promoter prior to LPS induction, but unphosphorylated CREB can bind CBP/p300 (32)

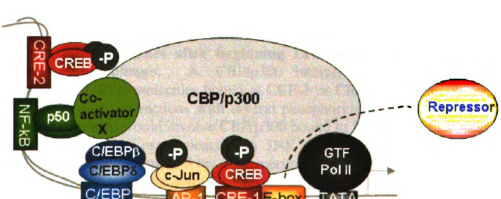
A Unstimulated



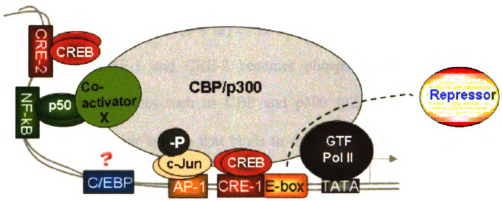
B 1 hr after LPS stimulation



C 4 hr after LPS stimulation



D 6 hr after LPS stimulation



E 8-12 hr after LPS stimulation

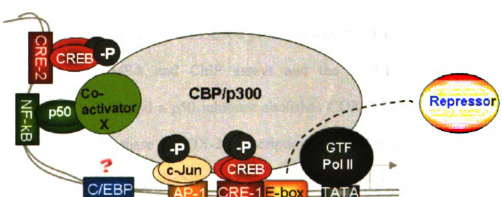


Figure 21. Model depicting the actions of transcription factors at the COX-2 promoter at different times after beginning LPS-induced COX-2 expression in RAW 264.7 macrophages. A. CBP/p300 interacts weakly with one of the unphosphorylated CREB molecules bound at CRE-1 or CRE-2. B and C. CBP/p300 is bound tightly through interactions with several phosphorylated transcription factors; an alternative arrangement would involve CBP/p300 bound to P-CREB via CRE-2. D. This formulation is based on experiments with JNK and p50 inhibitors which together completely block transcription at 6 hr. E. Experiments with JNK and p50 inhibitors and EMSAs indicate that promoter activity involves the NF-κB and AP-1 sites plus one or more additional sites such as CRE-1/2 and C/EBP.

and such an interaction involving one of the two CREs could recruit CBP/p300 and subsequently Pol II to the promoter.

During the early phase (0-4 hr) of LPS treatment (Fig. 21B), we speculate that CREB bound at CRE-1 and CRE-2 becomes phosphorylated which enhances the recruitment of coactivators such as CBP and p300 (32). Additionally, the 65/p50 heterodimer is the major species that binds to the NF-κB site and p65 is presumably phosphorylated, and the C/EBPβ homodimer binds to the C/EBP element (25). In the proximal region of the COX-2 promoter a heterodimer of newly phosphorylated c-Jun/c-fos becomes bound to the AP-1 site. Phosphorylated p65, C/EBPβ, and phosphorylated c-Jun have all been shown to interact with CBP/p300 (49-51). This suggests that the various activated transcription factors acting in concert may be recruiting CBP/p300 and general transcription factors to the COX-2 promoter leading to active transcription.

Based on EMSA and ChIP assays and the experiment showing that the combination of a JNK and a p50 inhibitor abolishes COX-2 induction at 4 hrs of LPS treatment, the middle phase of COX-2 transcription (4-8 hr, Fig 21C and 21D) appears to be dominated by the AP-1 and NF-κB sites and presumably involves phosphorylated c-Jun homodimers bound to the AP-1 site and p50 homodimers bound to the NF-κB site. p50 Homodimers are typically associated with negative regulation of gene transcription (52), but there are reports suggesting that p50 homodimers can act as transcriptional activators (53, 54).

The rate of COX-2 transcription increases at about 8 hrs of LPS after the middle phase of induction and remains elevated at 12 hr (Fig. 21E). During the late stage of induction (ca. 8-12 hr), there is increased phosphorylation of c-Jun such that the

concentration of phosphorylated c-Jun is increased and a relatively higher level of phosphorylated c-Jun homodimer binds to the AP-1 site. Binding of p50 homodimers to the NF-κB sites also occurs and at levels comparable to those seen during the middle phase of induction. The amount of CREB bound to the CRE-2 and CRE-1 elements remains the same before and throughout the LPS treatment, however CREB may be phosphorylated during the late phase of LPS stimulation. Binding of C/EBP transcription factors to the C/EBP site may also contribute to enhanced transcription during the late phase. Again, all these transcriptional activators are likely to be facilitating transcription by binding to CBP/p300.

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CHAPTER 3

TRANSCRIPTIONAL REPRESSION OF CYCLOOXYGENASE-2 THROUGH THE E-BOX ELEMENT IN LIPOPOLYSACCHARIDE-STIMULATED MACROPHAGE CELLS

Summary

The cyclooxygenase-2 (COX-2) enzyme is highly induced by cytokines and proinflammatory mediators such as bacterial endotoxin (lipopolysaccharide, LPS) in monocytes and macrophages, leading to increased production of proinflammatory prostaglandins that exert diverse actions in inflammation. COX-2 transcriptional activation in RAW 264.7 murine macrophages is rapidly upregulated upon LPS treatment and involves coordination of several *cis*-acting elements and *trans*-acting factors. As mentioned in chapter 2, we have used promoter analysis to demonstrate that the E-box element is involved in repression of COX-2 gene transcription in LPS-stimulated RAW 264.7 macrophages. Here, we examine the mechanism through which the E-box element of COX-2 mediates COX-2 transcriptional inhibition.

Using electrophoretic mobility supershift assays, we determined that the USF-1, USF-2, c-myc, and Miz-1 transcription factors are constitutively bound to the E-box and not further recruited in response to LPS treatment. Interestingly, overexpression of USF-1 or USF-2 did not suppress COX-2 promoter activity, suggesting that E-box mediated inhibition does not involve USF-1 or USF-2 even though they can bind to the E-box element of the COX-2 promoter.

Introduction

Cyclooxygenase (COX)-2 is one of two known COX isozymes (also known as prostaglandin endoperoxide H synthase, PGHS), which catalyzes conversion of arachidonic acid to prostaglandin H_2 (PGH_2), the precursor of prostaglandins, prostacyclin, and thromboxane (1). Arachidonic acid is released from membrane phospholipids in response to inflammatory stimuli such as LPS. Early in inflammation, newly transcribed COX-2 appears to be responsible for the production of prostaglandins whose overall function is to promote inflammation, fever, and pain (2).

In chapter 2, we described several *cis*-regulatory elements including a CRE-2 motif at -434/-428, an NF-κB element at -401/-393, a C/EBP site at -138/-130, an AP-1 site at -73/-61, and an overlapping CRE-1 and E-box element at -59/-48 that are involved in COX-2 transcriptional regulation. We further suggested that the E-box is involved in inhibition of COX-2 gene expression in LPS-treated RAW 264.7 cells.

The mouse COX-2 promoter contains a consensus E-box (5'-CACGTG-3') which is the principle binding site for members of the basic helix-loop-helix (b-HLH) and basic helix-loop-helix leucine-zipper (b-HLH-LZ) transcription factor families. The bHLH protein family is characterized by a basic DNA binding region adjacent to a helix-loop-helix dimerization region, both of which are required for the formation of functional DNA binding complexes (3). Upstream stimulatory factor (USF) is a bHLH-LZ protein that binds to the consensus E-box sequence (3). The bHLH-LZ transcription factor family also includes Myc (4) and Myc-subfamily factors Max (5), and Mad (6). There are two variants of USF. USF-1 and USF-2 are homologous genes which code for

structurally and functionally related proteins. Both USF proteins associate with E-box as homo- or heterodimers to regulate transcription of their target genes (3).

USF has been implicated in activating COX-2 expression in *Helicobacter pylori*-stimulated human gastric epithelial cells (7) and gonadotropin-treated granulosa cells (8). However, USF has been shown to act as a transcriptional suppressor in glutamate-cysteine ligase (GCLC) gene expression in rat lung epithelial L2 cells (9) and IL-1β-induced iNOS expression in mesangial cells (10). Another class of bHLH family members is the Myc transcription factors. Myc-family members such as Max, Mad, and Miz-1 function as dimers by interacting with a partner protein (11). Myc can both activate and repress transcription depending on the partner with which they heterodimerize. For example, the Myc/Max heterodimer activates transcription while the Myc/Miz-1 heterodimer inhibits transcription (11).

To investigate the mechanism by which the E-box plays a suppressive role in COX-2 gene expression, we identified transcription factors bound to the E-box of mCOX-2 promoter in LPS-treated RAW 264.7 macrophage cells. We also studied the role of USF in COX-2 inhibition. Although the E-box has proven to mediate transcriptional repression of COX-2 in RAW cells, we have determined that USF overexpression does not contribute to this suppression.

Materials and Methods

Antibodies. Miz-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibody to c-myc was purchased from Cell Signaling (Beverly, MA).

Plasmids. The pCMV-Miz-1, pCMV-Miz-1ΔPOZ, and pCMV-Miz-1 (Δ190-284) expression plasmids were kindly provided by Professor Martin Eilers (University of Marburg) (11).

Cell culture, transfections, luciferase reporter assays, electrophoretic mobility shift assays (EMSA), and all other antibodies and plasmids are described in the Materials and Methods section in chapter 2.

Results

Transcription factor binding to the E-box element in the mCOX-2 promoter. mCOX-2 promoter analysis including analysis of E-box mutations (Fig. 10) suggested that the E-box plays an inhibitory role in LPS-stimulated RAW 264.7 macrophages. To identify nuclear proteins bound to the E-box element, EMSA supershift assays were performed using a ^{32}P-labeled 25 bp double-standed DNA probe containing the overlapping CRE-1 and E-box element (Fig. 15 & Fig. 21). As was described in chapter 2, two distinct complexes were formed with the CRE-1/E-box probe (Fig. 21, lanes 1-3) and the amount of E-box protein complexes were not increased by LPS treatment. The higher mobility complex was shifted by antibodies to Miz-1 (Fig. 21, lanes 4-6), c-myc (Fig. 21, lanes 7-9), USF-1 (Fig. 21, lanes 10-12), or USF-2 (Fig. 22, lanes 13-15). These results indicate that the E-box is bound constitutively by Miz-1, c-myc, USF-1, and USF-2. The CRE-1/E-box complex that was not shifted by antibodies against the latter transcription factors involves CREB binding to CRE-1 (Fig. 15).

Effect of transcription factors bound to the E-box element on mCOX-2 promoter activity. EMSA supershift assays indicated that USF-1 or USF-2 binds to the CRE-1/E-box probe. To determine their influence on COX-2 transcription, USF-1, USF-2, or USF-1 T153A (a mutant missing a key phosphorylation site) expression plasmids were co-transfected into RAW 264.7 cells along with the COX-2 promoter luciferase construct (Fig. 22-24). Unexpectedly, USF-1, USF-2, or USF-1 plus USF-2 overexpression all caused a significant increase in luciferase activity (Fig. 22). This suggests that these factors are involved in activating COX-2 transcription even though

they bind to the E-box probe (Fig. 20) and are associated with the COX-2 promoter *in vivo* (Fig. 18). However, the extent of transcriptional activation by LPS did not change with USF-1 overexpression. Additionally, USF-1 T153A overexpression failed to increase luciferase activity to the level of wild type USF-1 or USF-2, indicating that the increase in COX-2 transcription by USF-1 requires T153 phosphorylation. Overexpression of USF-1 or USF-2 along with a luciferase reporter driven by a mutant E-box element of COX-2 promoter had the same activating effect as the wild type COX-2 promoter construct. These results indicate that USF-1 and USF-2 may not mediate COX-2 activation through the E-box, suggesting that there is an unidentified element within the COX-2 promoter that is responsive to USF transcription factors.

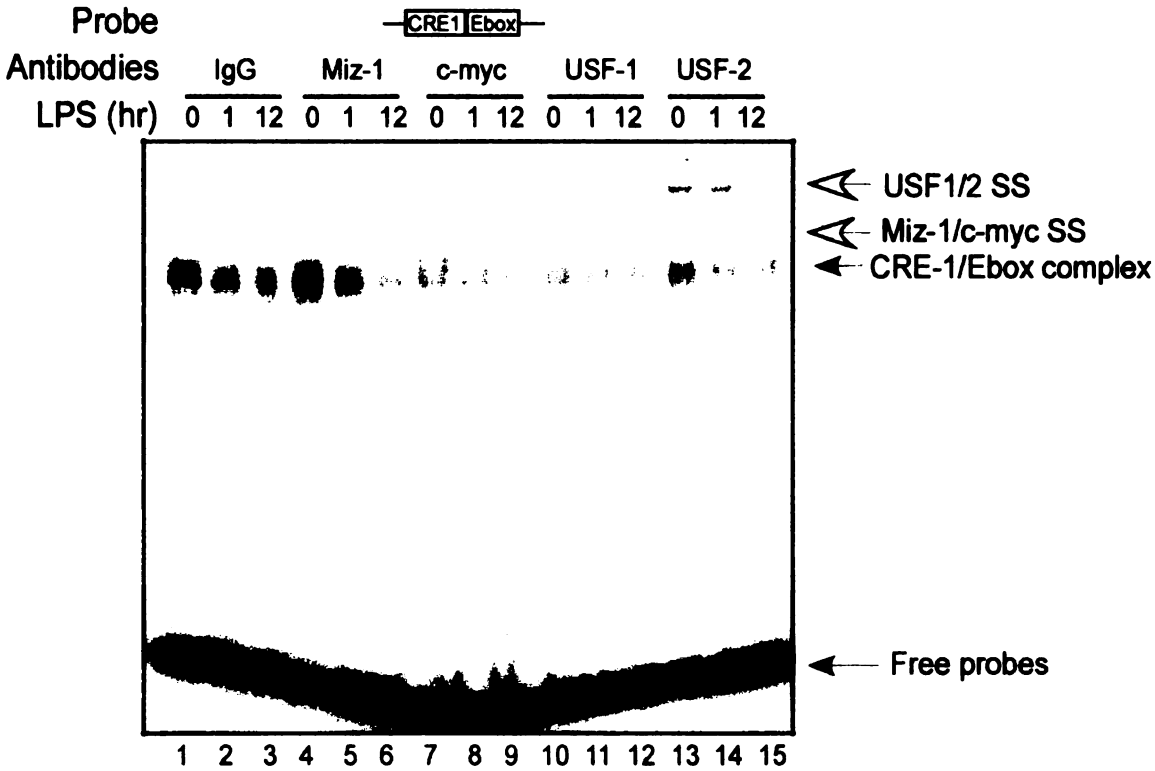
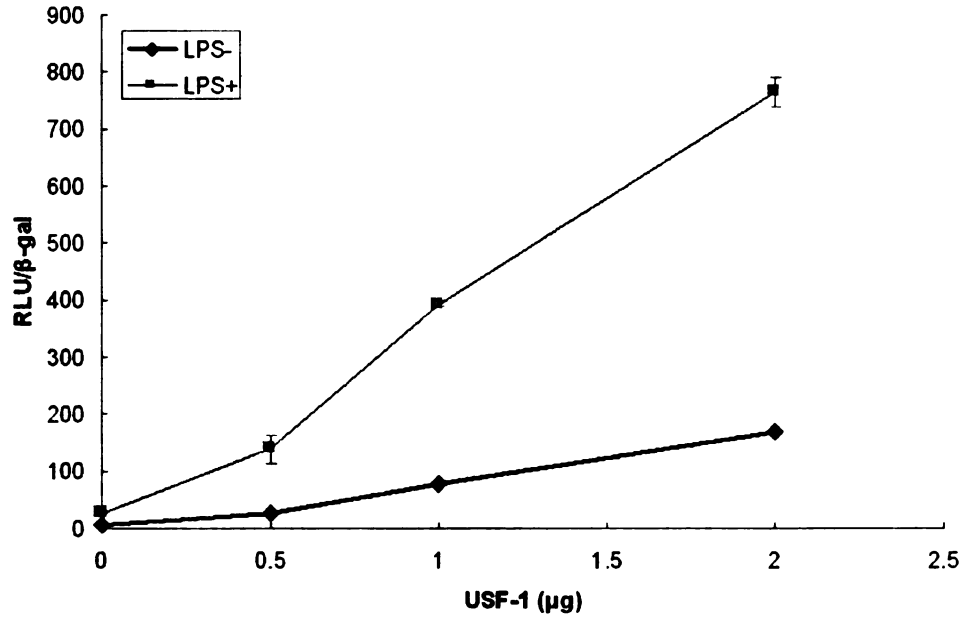
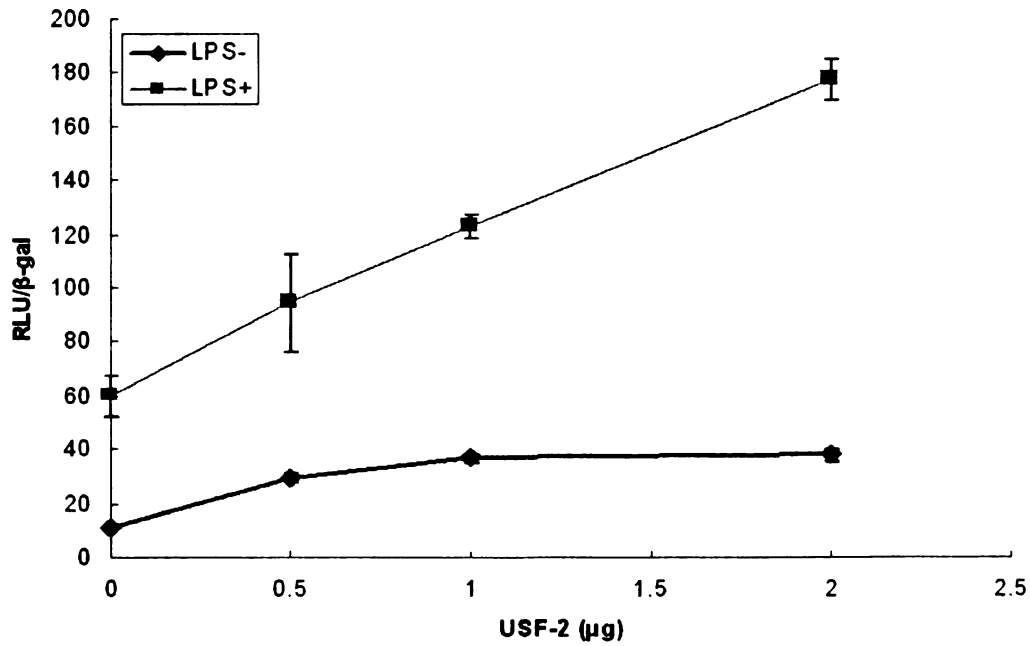


Figure 22. Transcription factor binding to the E-box probe. Nuclear extracts (5 μg) from RAW 264.7 cells treated without or with LPS for 1 or 12 hr were incubated with control IgG, Miz-1, c-myc, USF-1 or USF-2 antibodies, then a double stranded ^{32}P labeled 25 bp probe containing overlapping CRE-1 and E-box was added. Miz-1, c-myc, USF-1, and USF-2 supershifted complexes are indicated with open arrows and CRE-1/E-box complex and free probe are indicated with closed arrows.

A


LPS-
LPS+
RLU/β-gal
900
800
700
600
500
400
300
200
100
0
0
0.5
1
1.5
2
2.5
USF-1 (µg)

B


LPS-
LPS+
RLU/β-gal
200
180
160
140
120
100
80
60
40
20
0
0
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1
1.5
2
2.5
USF-2 (µg)

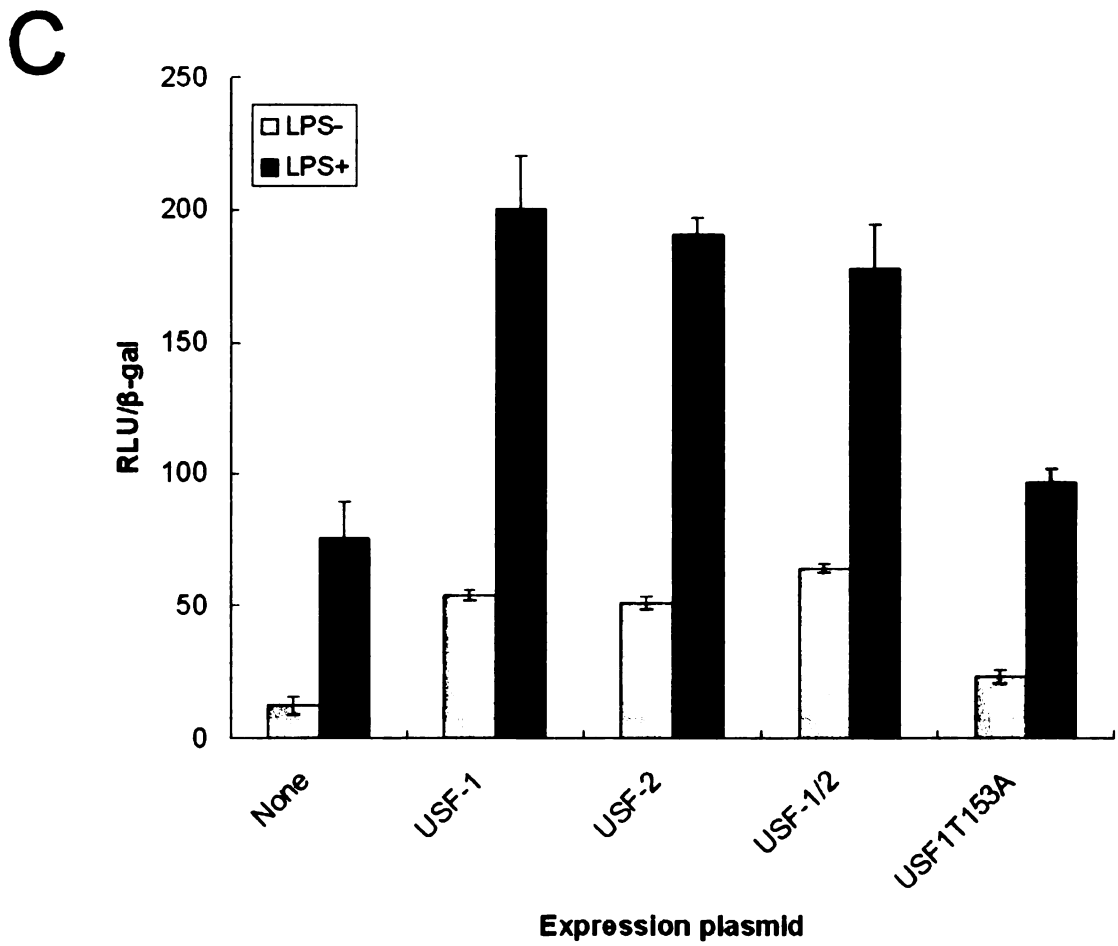

Figure 23. Effect of USF overexpression on LPS-induced COX-2 promoter activity. RAW 264.7 macrophages were transiently co-transfected with 1 μg of luciferase reporter driven by the COX-2 promoter the and indicated amounts of USF-1 (A), USF-2 (B), or 1 μg of USF-1, USF-2, USF-1/2, or USF1-T153A expression plasmids (C). At 24 hr posttransfection, cells were either not or treated with LPS for 12 hr. Luciferase activities were measured in cell lysates and normalized to β-galactosidase activities. Values represent the means of the results from duplicate samples. The results from a representative experiment of three independent transfections are shown.

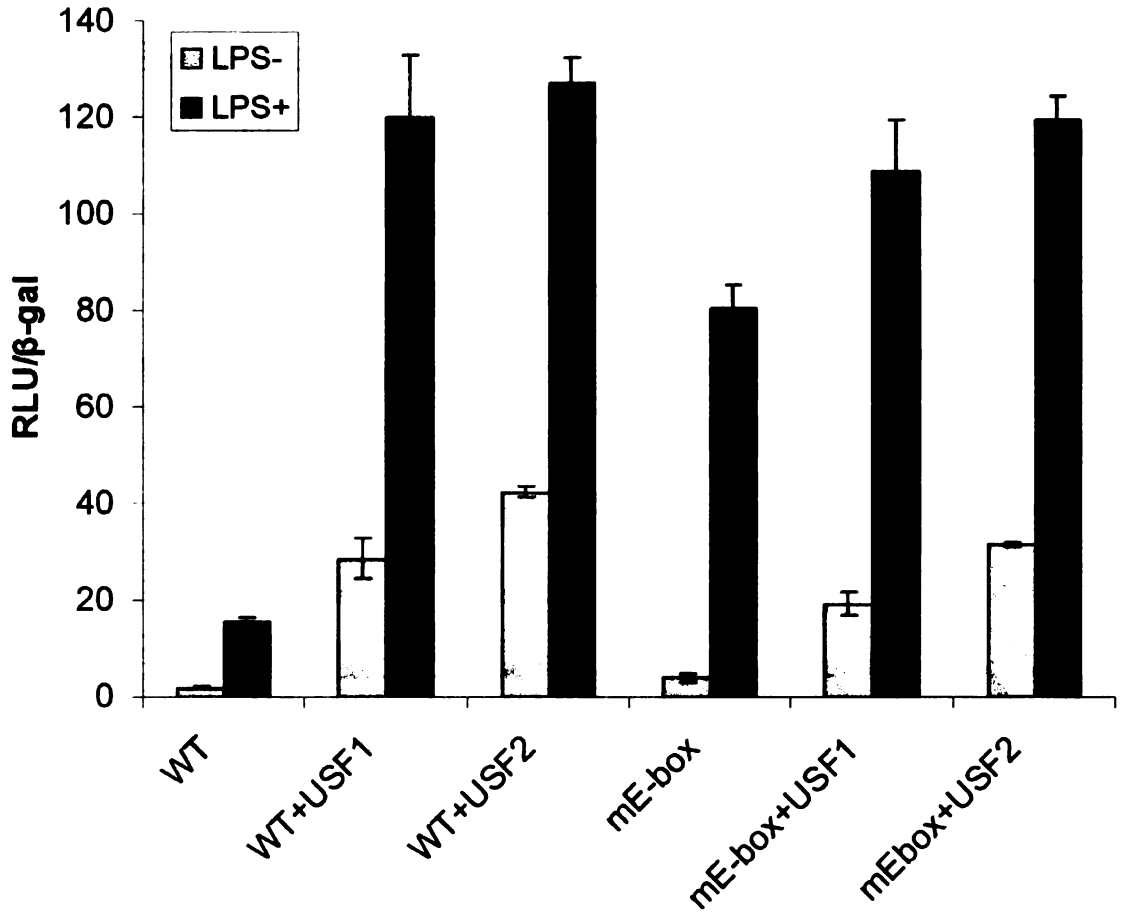

Figure 24. Effect of USF overexpression on LPS-induced WT or mutant E-box COX-2 promoter activity. A reporter construct driven by the WT COX-2 promoter or a mutant E-box COX-2 promoter having a mutated E-box were transiently co-transfected with empty vector, USF-1, or USF-2 expression plasmid into RAW 264.7 macrophage cells and incubated for 24 hr. LPS was added and cells were incubated for an additional 12 hr before being harvested and assayed for luciferase activity. Results of promoter assays are normalized to the expression of co-transfected β-galactosidase and are the average of two experiments in duplicate ±SD. The sequence of the E-box mutation is shown in Table III.

Discussion

In this study, we found evidence that the E-box element of the mCOX-2 promoter mediates transcriptional repression in LPS-stimulated RAW 264.7 cells. EMSA supershift assays revealed that the E-box element is bound by bHLH transcription family members, USF-1, USF-2, c-myc, or Miz-1. Consistent with this result, ChIP anlaysis showed that USF-1 and USF-2 are associated with the COX-2 promoter *in vivo* (Fig. 18). Binding of these proteins was observed with nuclear extracts from unstimulated RAW 264.7 cells and the level of binding did not increase or decrease after LPS treatment (Fig. 21). We speculated that USF-1 or USF-2 may function as a repressor by binding to the E-box element of COX-2 promoter. However, USF overexpression did not cause a decrease in COX-2 promoter activity as expected (Fig. 22). Contrary to our expectation, USF overexpression increased the luciferase activity in both basal and LPS treated samples by approximately 2.5 fold, respectively. The overall fold increases before and after LPS treatment did not change with or without USF overexpression.

USF-1 has a phosphorylation site at T153 which can be phosphorylated by various kinases including p38, protein kinase A (PKA), protein kinase C (PKC), cdk1, and PI3-kinase (12-15). While p38, PKA, PKC, and cdk1 kinase-mediated phosphorylation increases USF binding to the E-box, PI3K phosphorylation inhibits its binding to this element. In our overexpression studies, mutation of the USF-1 phosphorylation site (USF-1-T153A) abolished its ability to increase both basal and LPS-induced COX-2 promoter activities, indicating that USF-1 activation of COX-2 requires phosphorylation of T153.

Cotransfection assays with a mutant E-box COX-2 promoter construct (Fig. 23) suggest that USF-1 and USF-2 do not function through the E-box motif to activate COX-2 expression but rather may bind another region of the COX-2 promoter. Recently, USF-1 has been shown to associate *in vivo* with Fra1, a member of the b-Zip protein family, to promote transcription, demonstrating the cross-talk between distant members of the protein family (16). Thus, it is also possible that USF might interact with other transcription factors to increase transcriptional activity in our system.

Another potential mechanism by which the E-box motif may repress COX-2 expression in LPS-treated murine macrophage cells is through the Myc/Miz-1 transcriptional repressor complex. The Myc protein forms a binary activating complex with its partner protein Max. However, Max proteins may interact with the zinc-finger transcription factor Miz-1 to form a ternary repressor complex (11). Our EMSA supershift assays showed that c-myc or Miz-1 also binds to the E-box element of mCOX-2 promoter. The functional role of the Myc/Miz-1 transcription factor in LPS-stimulated RAW 264.7 cells still needs to be investigated.

The E-box motif of the COX-2 promoter has been reported to be involved in activation of COX-2 transcription through the USFs in other systems including human gastric epithelial cells by *Helicobacter pylori* (7) and granulosa cells by gonadotropin (8). However, the E-box as a repressor element in COX-2 gene expression had not previously been reported. Elucidation of the molecular basis for the differences between systems where the E-box acts as a positive regulator and those where the E-box as a negative regulator will greatly improve our understanding of COX-2 transcriptional regulation. Pharmaceutically modulating the factors associated with this bifunctional E-box element

may allow COX-2 gene expression to be regulated a cell or tissue specific fashion. For example, inhibiting the interaction between regulatory *trans*-acting factors and the E-box in macrophage would be expected to lead to increased COX-2 expression in response to LPS, but would likely lead to reduced COX-2 expression in granulosa cells during ovulation.

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