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THE TRANSCRIPTIONAL REGULATION OF PROSTAGLANDIN SYNTHASE-2 IN LIPOPOLYSACCHARIDE STIMULATED MACROPHAGE CELLS

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THE TRANSCRIPTIONAL REGULATION OF PROSTAGLANDIN SYNTHASE-2 IN LIPOPOLYSACCHARIDE STIMULATED MACROPHAGE CELLS

By

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ABSTRACT

THE TRANSCRIPTIONAL REGULATION OF PROSTAGLANDIN SYNTHASE-2 IN LIPOPOLYSACCHARIDE STIMULATED MACROPHAGE CELLS

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Prostaglandin H synthase (COX) catalyzes the first committed step in the metabolism of arachidonic acid to prostaglandins. There are two isoforms of COX, the predominantly constitutive isoform, COX-1, and an inducible isoform, COX-2. Although both enzymes catalyze the same reaction with similar kinetics, studies with isoform specific inhibitors and COX-1 and COX-2 knockout mice suggest that there are physiological processes that require one specific enzyme and others where both isoforms function together.

COX-2 expression is upregulated by a variety of stimuli in different cell types. Bacterial lipopolysaccharide (LPS) is a potent inducer of COX-2 activity in macrophage cells. LPS signaling is mediated through the toll-like receptor-4 and results in the activation of JNK, ERK, p38, NIK and PKC signaling pathways. COX-2 transcription is regulated through multiple redundant mechanisms involving their interactions with several central response elements. Characterization of the COX-2 gene promoter has resulted in the identification of *cis*-acting response elements that are necessary for maximal promoter activity in LPS-treated macrophage cells (Figure 2). The CRE at –57/-52 is necessary for mediating the effects of a wide variety of stimuli, while a pair of C/EBP sites 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 contain paired CRE and NF-κB

sites located approximately between 380 and 550 bp upstream of the transcription start site. Here we show that this conserved, upstream CRE (CRE-2) located at -434/-428 in the murine promoter is required for maximal induction of COX-2 by LPS in RAW 264.7 cells. Characterization of this site revealed that CREB/ATF transcription factors and the CREB binding protein from nuclear extracts of LPS-stimulated RAW 264.7 cells physically interact with this response element.

The NF-κB site is necessary for lipopolysaccharide induced COX-2 expression in bovine atrial endothelial cells and MC3T3-E1 osteoblast like cells, but has not previously been demonstrated as necessary for the LPS response in RAW 264.7 macrophage cells. Recently Rhee *et al.* demonstrated that blocking NF-κB activation at several levels also blocks induced COX-2 promoter activity. Potent reduction in COX-2 expression was also observed in experiments using decoy NF-κB, antisense expression, and inhibitors of IκB degradation. We found that the NF-κB response element was necessary for maximal promoter activity. While characterizing the Rel components that bind the NF-κB response element, we observed an unusual pattern of binding where the probe was initially bound predominantly with a p65/p50 heterodimer and then later by a p50 dimer. Since p50 dimers are generally considered transcriptional repressors, we measured the rate of COX-2 transcription and found that the prolonged COX-2 response was due to persistent transcription of the gene. Our promoter analysis data indicate that the CRE-2 and NF-κB act together to activate COX-2 transcription.

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LIST OF ABREVIATIONS

AP-1	Activator Protein-1
bFGF	basic Fibroblast Growth Factor
C/EBP	CAAT Enhancer Binding Protein
cAMP	Cyclic Adenosine Monophosphate
COX	Cyclooxygenase
CRE	cAMP Response Element
CREB	cAMP Response Element Binding Protein
Dex	Dexamethasone
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
HFF	Human Foreskin Fibroblast
I-κb	Inhibitor of κB
IKK	Inhibitor of κB Kinase
IL-1,2,6,8	Interleukin-1, -2, -6, -8
IRAK	Interleukin-1 Receptor Associated Kinase
JNK	c-Jun N-term Kinase
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MAPKAP-K1	MAPK- Activated Protein Kinase-1
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
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
PDGF	Platelet Derived Growth Factor
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PGG2	Prostaglandin G2
PGH2	Prostaglandin H2
PGHS	Prostaglandin H Synthase, Prostaglandin Endoperoxide Synthase
PGI2	Prostaglandin I2
PKA	cAMP dependent Protein Kinase
PKC	Calcium dependent Protein Kinase
PMA	Phorbol 12-Myristate 13-Acetate

PTH	Parathyroid Hormone
SSC	Salt-Sodium Citrate
TAE	Tris-Acetate EDTA
TAK-1	Transforming growth factor-beta Activated Kinase-1
TCF	T-Cell Factor
TGF-α -β	Transforming Growth Factor-α -β
TLR4	Toll-Like Receptor-4
TNF-α	Tumor Necrosis Factor-α
TPA	Phorbol 12-Tetradecanoate 13-Acetate
TRAF	Tumor Necrosis Factor Receptor Associated Factor

CHAPTER 1

LITERATURE REVIEW

Introduction

The major groups of eicosanoids are the prostaglandins, prostacyclins, thromboxanes, leukotrienes, and epoxy acids. These biologically active lipids are synthesized primarily from arachidonic acid, which is a component of cellular membranes and is synthesized from the essential fatty acid linoleate or obtained through the diet. The prostanoids are the products of prostaglandin endoperoxide H synthase (COX); leukotrienes are products of the 5- lipoxygenase pathway, and epoxy acids are the metabolites of cytochrome P450s.

The prostanoids mediate a wide range of normal physiological responses. Pain and inflammation are mediated by prostaglandins. In animal models, joint inflammation as well as edema and hyperalgesia are blocked by COX inhibitors [1]. Prostaglandins sensitize the free ends of neurons and act centrally to increase general sensitivity to pain [2]. Blood clotting is mediated by the release of thromboxane from platelets. Inhibition of platelet aggregation by the COX inhibitors has lead to the concept of using half an aspirin tablet a day as a prophylaxis against thromboembolitic disease [3]. Prostaglandins are necessary at multiple steps in the reproductive process. In mice with knocked out prostaglandin receptor or COX, genes exhibit reproductive failures because of problems with ovulation, fertilization, implantation, decidualization, and parturition. In addition,

neonatal mice have severe renal pathology, malformed kidney structures, and patent ductus arteriosus [4, 5].

In the kidney, prostaglandins mediate glomerular hemodynamics and tubular reabsorbtion of water and sodium, and in the stomach low levels of prostaglandins inhibit acid and fluid release from the mucosal layer [6]. Higher levels of prostaglandins induce the secretion of acid and fluid from the lining on the stomach and gastrointestinal tract. Smooth muscle contraction and relaxation are mediated by prostaglandins. In the intestines and uterus, prostaglandins mediate contraction of longitudinal muscle and contraction of circular muscles [6]. Prostacyclin is involved in the maintenance of vascular tone and also functions in the circulatory system as an inhibitor of platelet aggregation[6]. Prostaglandins directly mediate the absorption and formation of bone tissue but are also indirectly involved in bone metabolism by affecting the differentiation and proliferation of osteoclast and osteoblast precursor cells [7].

There are two COX isozymes, the constitutive COX-1 and the inducible COX-2. COX-1 is expressed in most cell types and is involved in homeostasis and various physiological functions such as platelet aggregation, water and sodium metabolism in the kidney, stomach acid secretion, and parturition [8]. COX-2 was initially cloned as an immediate early gene, and its expression is rapidly upregulated in response to pro-inflammatory stimuli such as bacterial lipopolysaccharide (LPS), Tumor Necrosis Factor-α (TNF-α), Interleukins-1 and -2 (IL-1, IL-2), reactive oxygen species, hypoxia, and mitogenic stimuli [9]. COX-2 is upregulated up to 80 fold, often from nearly undetectable levels in many tissues and cell types. COX-2 is generally not found under normal physiological conditions; however, it is constitutively expressed in specialized

cells of the kidney and in brain and bone tissues. COX-2 knockout mice develop malformed kidney structures and severe renal pathology [4, 5]. In addition, the ductus arteriosus does not close in about one third of the cox-2$^-$/cox-2$^-$ mice [10].

The wide variety of physiological actions affected by the prostanoids require a broad range of cellular signaling capabilities, which are mediated by the prostanoid receptors. The prostaglandin receptors have been characterized pharmacologically using radioactive ligands, and the binding properties indicated that a variety of prostaglandins cross-react with more than one receptor, suggesting that the receptors share a high degree of structural similarity [11]. Biochemical studies have demonstrated that the actions of prostaglandins are mediated by G proteins resulting in changes in second messenger levels that are summarized in Table I. The receptors are classified by their primary agonist or antagonist into five groups termed DP, EP, FP, IP, and TP based on their sensitivity to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2, respectively. The EP receptors are further divided into four subgroups, EP1, EP2, EP3, and EP4, by pharmacological characterization with specific agonists.

Because of the second messengers released in response to the prostanoids, one prostaglandin may cause opposite affects in different tissues, or even within the same tissue. In the lining of the stomach, both the EP3 and EP4 receptors are found in similar tissue and are involved in the balancing the release of chloride ions into the stomach. The EP3 receptor has a higher affinity for PGE_2 than the EP4 receptor, so that at low ligand concentrations EP3 (coupled to phosphodiesterase to decrease intracellular cAMP) inhibits the secretion of acid, and at higher ligand concentrations EP4 (coupled to adenylate cyclase to increase cAMP) promotes acid secretion [12]. In other cases,

Table I. Classification of prostaglandin receptors and their signal transduction.

Data obtained from Narumiya *et al.* [13]

Ligand	Type	Subtype	Isoform	G protein	Signal transduction
PGD_2	DP			G_s	cAMP increase
PGE2	EP	EP_1		G_q (?)	PI response, Ca^{2+}
		EP_2		G_s	cAMP increase
		EP_4		G_s	cAMP increase
		EP_3	EP_{3A}	G_i	cAMP decrease
			EP_{3B}	G_s	cAMP increase
			EP_{3C}	G_s	cAMP increase
			EP_{3D}	$G_{i/s/q}$	cAMP decrease / increase, PI response
$PGF_{2\alpha}$	FP				PI response
PGI_2	IP				cAMP increase, PI response
TXA_2	TP	TP α		$G_{i/q}$	cAMP decrease, PI response
		TP β		$G_{s/q}$	cAMP increase, PI response

different prostanoids mediate opposing effects. Thromboxanes function as potent vasoconstrictors and mediate platelet activation and aggregation, and PGI_2 functions in an opposite role as a vasodilator and an inhibitor of platelet aggregation [6].

Localized expression of the receptors has been studied in cells and in tissues by Northern blot and in situ hybridization. Combined with pharmacological studies using cyclooxygenase inhibitors and various receptor agonists and antagonists, the physiological functions of the receptors have been defined. To clarify the role of each receptor and their ligand, receptor genes have been individually disrupted. The major phenotypes of mice deficient in prostanoid receptors are summarized in Table II. The knockout mice all grow normally with a few exceptions. The FP null mice die in utero because the mother does not go into labor when the pre-natal mice reach term. EP4 null mice die within 72 hr due to patent ductus arteriosus, and EP2 null females are defective in ovulation and fertilization [2].

Table II. Summary of prostaglandin receptor gene knockout data.

Data obtained from: Narumiya *et al.* [2] and Sugimoto *et al.* [14].

Disrupted Gene	Major Phenotypes of knockout mice
DP	Reduced responses in allergic asthma
EP1	Reduction in carcinogen-induced colorectal neoplasia
	Reduction in allodynia (tactile pain)
EP2	Impaired ovulation and fertilization
	Salt-sensitive hypertension
	Loss of bronchodilation
	Reduction in vasodepressor response to PGE2
	Reduced osteoclast generation
EP3	Impaired febrile response to pyrogens
	Enhanced vasodepressor response to PGE2
	Impaired duodenal bicarbonate secretion
	Reduction in Hyperalgesia (sensitivity to pain)
EP4	Patent ductus arteriosus
	Impaired vasodepressor response to PGE2
	Decreased inflammation bone resorption
FP	Parturition
IP	Thrombotic tendency
	Decreased inflammatory swelling
	Decreased acetic acid writhing
TP	Bleeding tendency, resistance to thromboembolism

The Role of Prostaglandins in Pain and Inflammation

Prostanoids are involved at multiple levels of the inflammatory response as well as chronic inflammation [2, 15]. At a very basic level, the research presented in this dissertation is intended to contribute to the understanding of how COX-2 is expressed in response to inflammatory stimuli. Inhibition of prostaglandin synthase enzymatic activity can greatly reduce the amount of pain and inflammation experienced as a result of trauma and tissue damage [16]. Another class of drugs, glucocorticoids, work in part by restricting the production of COX-2 and are highly effective in reducing inflammation; however the side effects of this therapy prevent its continuous use [17, 18]. If the transcriptional regulation of COX-2 is understood with enough detail, it may be possible to block the production of the enzyme with a specific, non-steroidal, transcription inhibitor.

Inflammation is a component of the innate immune response and is characterized by redness of the skin, swelling of the affected area, heat, and pain [16]. The initial phase of an inflammatory response includes vasodilatation and increased vascular permeability and is followed by the infiltration of neutrophils and monocytes. Arachidonic acid metabolites play a number of roles in this response, from leukotrienes that function as chemotactic agents that increase vascular permeability, to prostaglandins that act as vasodilators, chemotactic agents, and enhance the effects of histamine and kinins. The link between pain caused by the inflammatory process and prostaglandins was demonstrated by *in vivo* carrageenan rat paw experiments, which demonstrated that COX-2 selective inhibitors block edema and hyperalgesia following the inflammation inducing insult [19].

Prostaglandins play a direct role in sensing pain [14]. Prostaglandins sensitize the free ends of pain neurons and increase general sensitivity to pain [2]. These types of pain are referred to as hyperalgesia and allodynia. Independently, exogenous PGI_2 and PGE_2 can cause pain and edema, and *in vivo* PGE_2 antibodies block carrageenan induced hyperalgesia [20]. To mimic allodynia, PGE_2 is injected into the subarachnoid space between the lumbar vertebrae. As a result, normal mice respond to touch with a paintbrush as though it were noxious stimuli, by squeaking and biting at the paintbrush .

When the IP receptor gene is disrupted, mice no longer respond to pain caused by the intra-peritoeneal injection of acetic acid and are much less sensitive to the pain caused by heat in hot-plate studies [2]. EP1 and EP3 knockout mice exhibit decreased levels of inflammation induced pain [21], and in the EP3 mice, carrageenan induced inflammation was greatly reduced. These experiments provide evidence that prostaglandins mediate specific types of pain, sensitivity to pain, and generation of edema associated with painful inflammation.

Prostaglandins may not always function as mediators of pain and inflammation. Using the carrageenan induced inflammation model, Gilroy *et al.* [22] examined the contents of the fluid exuded from the pleural cavity of treated rats. They observed an immediate burst of COX-2 expression (in the cells found in the exudate) that correlated with high levels of PGE_2 and increasing levels of exudate volume. The resolution of the response is correlated with a decreased rate of fluid generation, infiltrating monocytes with high COX-2 activity, and a shift from PGE_2 production to high levels of PGD_2 and its metabolite 15-deoxy $\Delta^{12\text{-}14}PGJ_2$ [22].

In this *in vivo* model, prostaglandins and high levels of COX-2 expression appear to be at least correlated with anti-inflammatory actions. The 15 deoxy $\Delta^{12\text{-}14}PGJ_2$ prostaglandin product is associated with anti-inflammatory actions as a PPARγ ligand and an inhibitor of NF-κB activation. In addition, COX-2 inhibition has also been observed to negatively impact the healing of lesions in the mucosal layer of the stomach [23]. These papers suggest that under certain conditions, tissue specific COX-2 expression may play an anti-inflammatory role.

Catalysis of Arachidonic Acid to Prostanoids

Arachidonic acid is released from the cellular membranes by hydrolysis from glycerophospholipids by secretory or cytoplasmic phospholipase A_2 (PLA_2). In the first step of catalysis, arachidonic acid and two molecules of oxygen are converted to prostaglandin G_2 (PGG_2) through the cyclization of five central carbons of the twenty carbon chain. In the second step, PGG_2 hydroperoxide is reduced to the final product, prostaglandin H_2 (PGH_2). (Figure 1) The cyclooxygenase and peroxidase activities are located at different positions on the enzyme [9].

The membrane binding domain of the enzyme is comprised of four α-helical domains arranged end to end forming the opening of the cyclooxygenase site. When the substrate is completely inside the active site, the ω end of the arachidonate is buried within a highly hydrophobic pocket at the top of the channel, and the terminal carboxyl moiety interacts with polar and charged residues near the opening of the active site. The heme group of the peroxidase site is oxidized, which generates a tyrosine radical that abstracts a hydrogen from Carbon (C) 13 of arachidonic acid in the cyclooxygenase site. This arachidonyl radical reacts with a biradical oxygen molecule to form an endoperoxide bridge between C-11 and C-9. Further intra-molecular rearrangement of the radical results in the addition of another molecule of oxygen forming a hydroperoxide at C-15. The second step of the reaction occurs in a cleft on the top side (relative to the membrane) of the enzyme. Through a yet undiscovered mechanism, PGG_2 leaves the cyclooxygenase site and enters the peroxidase site where the reduction of the hydroperoxide at C-15 occurs. Initial activation of the heme group located between the peroxidase and the cyclooxygenase site requires a lipid peroxide-dependent oxidation.

The oxidized heme oxidizes the tyrosine, generating the tyrosine radical necessary for the cyclooxygenase activity [9].

Only one peroxidase turnover is required because the tyrosyl radical is regenerated independently of the peroxidase after each cyclooxygenase turnover. The cyclooxygenase continues to turn over until the enzyme suicide inactivates through an undefined autocatalytic mechanism in which the radical is transferred to an inappropriate residue that results in internal crosslinking and inactivation of the enzyme [9, 24].

The COX-1 and COX-2 enzymes share approximately 60% primary sequence identity [17], and their protein crystal structures are nearly identical; however, there are several small differences in the substrate binding domain and active site. The opening of the COX-2 active site is approximately 20% larger than COX-1. This is due to a change from an isoleucine in COX-1 to a valine in COX-2 as well as several changes in the secondary shell to residues with smaller side chains. As a result, an arginine residue at the opening of the active site that is critical for stabilizing the carboxylate of arachidonic acid in COX-1 is displaced [9]. The increased size of the opening and hydrophobic side pocket are the discriminating factors for COX specific inhibitors [3].

COX-1 is irreversibly inhibited by aspirin by the acetylation of serine 530 forming a prominent protrusion in the opening of the channel, and this is thought to prevent the entrance of arachidonic acid to the active site [17]. Acetylated COX-2 still forms prostanoid products; however, because of the misalignment of C-13, most of the products formed have only the C-15 hydroperoxide but not the bicyclic peroxide. The larger more flexible substrate channel and small internal pocket have been

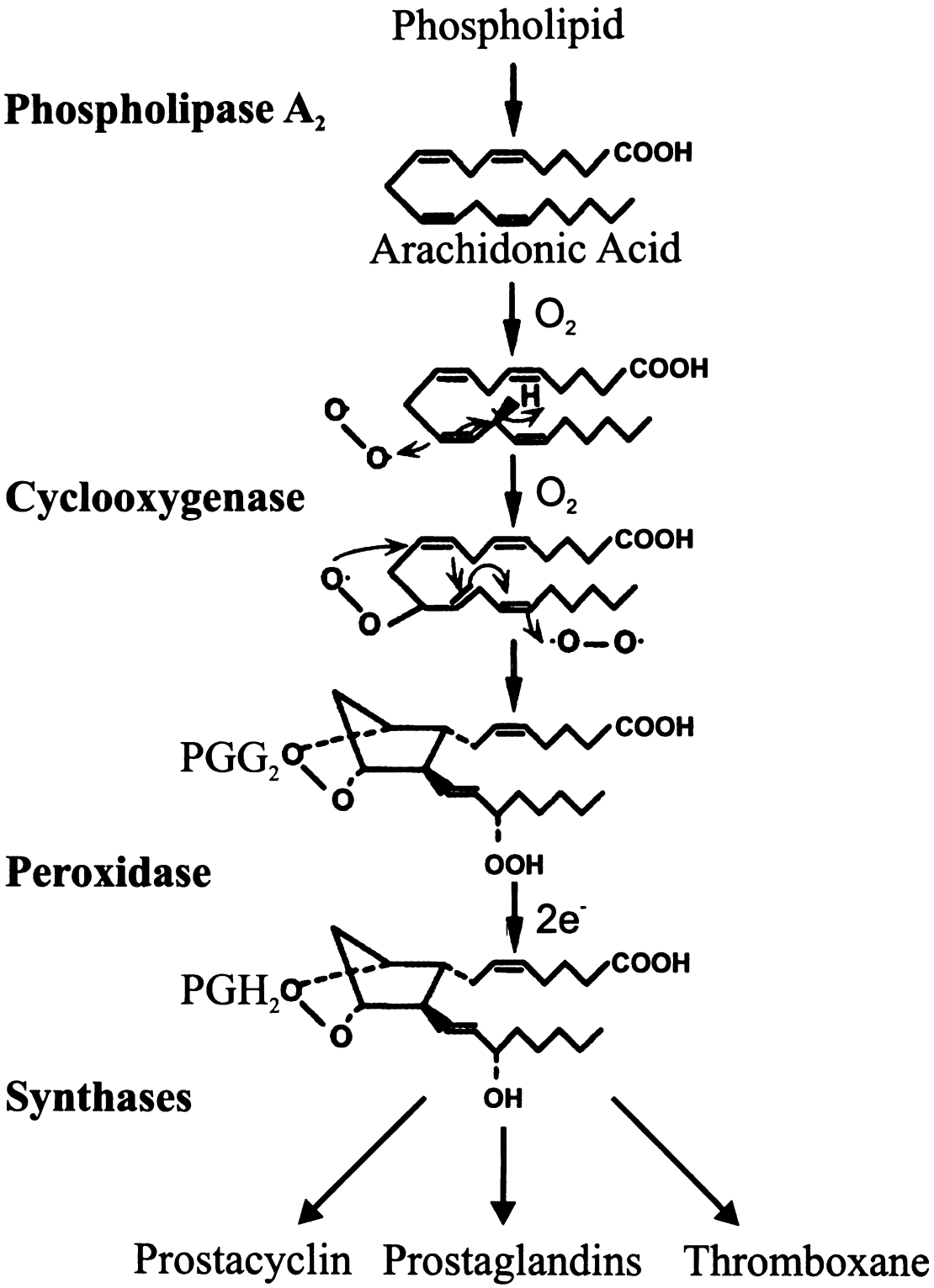

Figure 1. Arachidonic Acid Metabolism

exploited for the development of COX-2 selective inhibitors such as rofecoxib (Vioxx®) and celecoxib (Celebrex®) whose structures occupy this unique side pocket.

Both enzymes carry out identical catalytic actions, so why are there two isozymes? One hypothesis is that the COX isozymes are part of discrete biosynthetic pathways involving the coupling of distinct pools of arachidonic acid, specific phospholipases, and downstream prostaglandin synthases. There are at least 16 PLA_2 proteins that are grouped by size, substrate specificity, calcium dependence, and structural homology and fall into three general categories [25]. The cytoplasmic phospholipases ($cPLA_2$) are calcium dependent and arachidonic acid specific; the secretory phospholipases ($sPLA_2$) are also calcium dependent but are not specific for arachidonic acid, and the intracellular PLA_2s ($iPLA_2$) are neither calcium dependent nor arachidonic acid specific. Nearly any stimulus that activates MAP kinase signaling or elevates intracellular calcium concentrations is sufficient to activate the $cPLA_2$s [26]. Activated $cPLA_2$ translocates from cytoplasm to the exterior of the endoplasmic reticulum where it specifically releases arachidonic acid from the membrane. The initial burst of prostaglandin production, 10 to 60 min post treatment, is metabolized by the constitutive COX-1, and the delayed prostaglandin production is a result of newly synthesized COX-2. Early studies suggested that different pools for arachidonic acid and distinct PLA_2s were functionally if not physically linked to either COX-1 or COX-2. The $sPLA_2$ is thought to be coupled to COX-2 in the late phase of prostaglandin production; however, this may be due to the temporal expression of $sPLA_2$ and the specific activity of COX-1 and COX-2 at low substrate concentrations. *In vitro*, at high substrate concentrations, both enzymes have identical Km values [9]. However, *in vivo* and at very

low substrate concentrations (0.05 – 2 μM) the apparent Km values of the two isoforms appear to be different. This is because the Km is influenced by peroxide concentrations. COX-1 requires peroxide concentrations that are about 10 times greater than those of COX-2. Because of these two effects, arachidonic acid is preferentially metabolized by COX-2 when there are low concentrations of arachidonic acid present. [3, 6, 8, 9, 17, 27-29].

Physical interactions between the PLA_2s and downstream synthases have not been observed, but since these proteins localize to the membrane of the endoplasmic reticulum, it is possible that weak interactions do exist [30] [9]. The recently identified prostaglandin E_2 synthase (PGES) [31] isozymes appear to be functionally coupled to specific COX isozymes. The cytosolic PGES is constitutively expressed in many cell types and appears to be coupled to COX-1 in the early phase of prostaglandin synthesis. In contrast the membrane-associated PGES is inducible by inflammatory stimuli and appears to be coupled to COX-2 in the late phase of prostaglandin production [32, 33]. In response to inflammatory stimuli, there appears to be a shift from the production of thromboxane B_2 (TXB_2), prostaglandin D_2 (PGD_2) and prostaglandin I_2 (PGI_2) to increased levels of prostaglandin E_2 (PGE_2) and PGI_2 [34-37].

Historical Perspective

As early as 1975, Lawrence Levine began using methylcholanthrene-transformed BALB/3T3 fibroblasts in an attempt to study the mechanisms of initiation of the biosynthetic process resulting in prostaglandin production. His idea was that cells grown in culture might be a simpler model than using subcellular fractions of tissues, intact organs, or tissue slices. Within a short time, Levine discovered that prostaglandin production could be enhanced by serum or phorbol-ester (TPA) stimulation, and that these "stimulations" were dependent on protein and mRNA synthesis [38-40]. Inducible COX activity was also characterized in response to growth factors, LPS, and IL-1. In retrospect, these detailed studies were the first to characterize inducible COX-2 activity. In some cases, the use of COX-2 cross reactive antibody even allowed the characterization of induced COX-2 protein in tissues with very low constitutive COX activity [41-43].

The biochemical identification of a second COX activity was published by Robert Gorman's lab at the Upjohn company in Kalamazoo, Michigan. Alice Lin *et al.* reported that Platelet Derived Growth Factor (PDGF) stimulated, serum starved fibroblasts resulted in bursts of PGE_2 synthesis that began 10 min post stimulation and peaked after 2 hr. NIH3T3 cells constitutively expressed COX, and PDGF increased COX mRNA levels after 2 hr even though protein levels remained nearly constant throughout the experiment [44]. Gorman observed that arachidonic acid treated, serum starved NIH3T3 cells could synthesize PGE_2 in the absence of mitogen stimulation even more rapidly and potently than when stimulated with PDGF, and that unstimulated arachidonic acid dependent PGE_2 production could be blocked with aspirin pre-treatment. In the aspirin

treated cells, arachidonic acid stimulated PGE_2 synthesis was not recovered. This implied that the cells that constitutively expressed a COX activity could be blocked by aspirin. Pretreatment with aspirin (an irreversible COX-1 inhibitor) and stimulation with PDGF resulted in a PGE_2 response that took two hr to develop and peaked at three hr. In these cells, cyclohexamide[1] completely abolished the delayed synthesis of PGE_2. While several very rational explanations were postulated for their observations, the authors also speculated wildly that "PDGF induces the expression of a second PGHS[2] that is coupled to the PDGF receptor and whose mRNA is not readily detected by our probe. Thus, the possibility remains that there is differential mRNA splicing or even a second gene."

In the late 1980s a number of labs were hoping to find a cancer cure using the power of molecular biology. To this end, a technique called "subtractive and differential screening" was employed to identify nuclear targets for mitogenic signal transduction pathways. Shortly after mitogenic stimulation, immediate-early genes were induced, causing rapid increases in their mRNA. Since up-regulation of these genes didn't require prior protein synthesis, they were presumed to be the necessary requirements to drive quiescent (G_0) cells into the first stage of the cell cycle (G_1). Many of the genes first identified were transcription factors or secreted proteins, but most of the cloned genes had no known function [45, 46]. Sequencing and characterization of the gene products were the rate-limiting steps for discovery.

In November of 1988, Daniel Simmons *et al.* published his work on genes with unknown functions (or sequences) that were induced in a temperature sensitive Rous Sarcoma Virus (v-Src) infected chicken embryo fibroblasts (CEF), that could also be

[1] Cyclohehamide is a protein synthesis inhibitor and is used to determine if an enzyme activity is the result of proteins that are currently in the cells or if it is the result of a newly synthesized protein.

induced by TPA, and serum stimulation in normal CEF [47]. Simmon's CEF clone 147 mRNA was nearly undetectable in resting cells and was induced by one hr, peaking at two hr at "superinduced" levels with an apparent size of about 5 kb. Its function was unknown and was one of 6 clones awaiting further characterization.

Within months of Gorman's observation of a second inducible COX activity, Glenn Rosen *et al.* in Michael Holtzman's lab at the University of Washington (St. Louis, Mo.), observed that induced COX activity that didn't correlate with either the COX-1 protein levels or with the 2.8 kb COX-1 mRNA expression patterns in sheep tracheal epithelial cells. Northern blotting at low stringency conditions using two non-overlapping COX-1 probes revealed a 4 kb mRNA that was expressed basally at very low levels, was tissue specific, and whose expression pattern followed the increase in COX enzyme activity. They hypothesized that the 4 kb mRNA was derived from a distinct COX related gene. Curiously, the Northern blots from the Gorman lab, which had a number of non-specific bands, had one that appears to be about 4 kb and follows the pattern of PGE_2. Rosen *et al.* set the standard for the identification of the proposed COX-2, saying that although the 4 kb mRNA likely represents an explanation of their excess COX activity, "verification that the larger mRNA encodes for a cyclooxygenase will require molecular cloning of the gene and expression of a functional protein product in cells lacking endogenous activity." [48]

Jia-Wen-Han et al, from the labs of Donald Young and Ian Macara (University of Rochester, NY), published a paper on the persistent induction of cyclooxygenase in v-Src transformed BALB/c 3T3 fibroblasts [49]. Their model for oncogenic transformation was based on the idea that either cells were transformed by the expression of transformation

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[2] Prostaglandin endoperoxideH synthase (PGHS)

specific genes that are not normally expressed, or they were a result of continued expression of genes that are only transiently induced during mitosis. Instead of using differential subtractive hybridization, they used "giant two-dimensional electrophoresis" to look for rapid changes in protein abundance after activation of a temperature sensitive Rous sarcoma virus infected fibroblast. What they thought they found was a post-translational modification of the COX-1 gene product. This was a unique finding: out of >3000 polypeptides resolved by their method, this was the only one that appeared as a doublet and was inducible. We now know that COX-1 has an apparent molecular weight of 72 kDa, and that COX-2 generally is separated as two bands that are 72 and 74 kDa as a result of incomplete glycosylation. The reason BALBc 3T3 fibroblasts were used was because they have only a very low background COX-1 expression. In contrast, NIH3T3 fibroblast cells have a relatively high constitutive level of COX-1. Since dexamethasone and indomethacin treatment did not cause reversion of the fibrosarcoma, but dexamethasone blocked COX-1 expression and indomethacin blocked PGE_2 synthesis, the authors concluded that a post-translational modification was probably involved with the role of COX and its function in its unregulated state. Others suggested that post-transcriptional modification was behind the mechanism of the differential regulation of induced COX gene expression, based primarily on the ability of dexamethasone to block serum induced expression without affecting enzyme activity, mRNA abundance, or the presence of the 72 kDa protein species.

On the day after Christmas in 1990, Daniel Simmons, at Brigham Young University, communicated an exciting find to PNAS. Weilin Xie *et al.* had sequenced their CEF-147 clone and discovered that it was 59% identical to the ovine COX-1 [50]. *In*

vitro transcription of their 4.1 kb mRNA resulted in a 70 kDa protein, and co-translational glycosylation produced a 79 kDa protein. This was comparable to the predicted 68 kDa ovine COX-1 and the potentially novel 74 kDa protein that had been observed. There was one significant problem. COX-1 had been cloned and sequenced from ovine, murine, and human sources, but not from chicken. Was this a closely related protein or the chicken homolog to ovine COX? The authors argued that they had cloned the inducible COX gene and cited several very significant differences between their gene and the COX-1 gene. First, this new gene was post-transcriptionally regulated by the splicing of an intron in the 5' region of the mRNA that blocks translation. Second, the new gene had an unusually long 3' untranslated region (UTR). The 3' UTR makes the transcript 4.1 kb long instead of the usual 2.8 kb COX-1. The fibroblast experiments of Rosen *et al.* had implicated a 4 kb mRNA as a possible COX-1 related gene product in mouse fibroblasts. Weilin Xie *et al.* [50] also noted that the 3' UTR also contained a number of Shaw Kamen RNA instability sequences that are not present in the cloned COX-1 3' UTRs. This data certainly implied a possible homology to the 4 kb mouse mRNA, which helped to suggest the existence of two COX isoforms, but until the mouse transcript was cloned, the identity would still be uncertain.

While Simmons had been using CEF for his subtractive differential screening experiments, Harvey Herschman at UCLA (Los Angeles, CA) was using Swiss 3T3 fibroblast cells and was screening through his own group of induced immediate early genes with unknown functions. Dean Kujubu *et al.* reported that they had cloned a number of TPA inducible genes [51]. TPA inducible sequence #10 (TIS-10) was induced by TPA, forskolin, and serum in Swiss 3T3 cells. The expression of TIS-10 appeared to

be cell type restricted, in contrast to most of the other immediate early genes they had identified, but its function was unknown.

The rate of progress in characterizing the new COX related transcript moved very quickly in 1991. By July, the Donald Young lab from Rochester submitted a publication in which they correlated serum stimulated and glucocorticoid regulated levels in both immunoblot and immunoprecipitated COX with an abundance of the 4 kb mRNA species visualized with low stringency washing of the COX-1 probe [52]. They reported cloning the inducible COX cDNA and published an 80 amino acid translation of their preliminary sequence data, which was 95% identical to the translated sequence published by Simmons and was 59% identical to ovine COX-1.

JoAnne Richards produced polyclonal antibodies that recognized an inducibly expressed and tissue restricted COX enzyme and was able to show that the different molecular weight variants of COX observed in rat ovaries were antigenically distinct. Using antibody against a distinct prostaglandin synthase, Jean Sirois *et al.* purified and determined the amino-terminal sequence of a peptide that was nearly identical to the translation of the previously identified TIS-10 and CEF-147 sequences from mouse and chicken cells, respectively [53, 54].

Bradley Fletcher *et al.*, from Herschman's lab, published a second paper in October of 1991 that included the gene structure of COX-2 and expression data showing that the TIS-10 gene conferred cyclooxygenase and peroxidase activity on transiently transfected COS cells [55]. They also fused several portions of the genomic DNA 5' of the transcriptional start site to a luciferase reporter plasmid to show that the luciferase activity could be induced in a pattern similar to that of the new COX in TPA stimulated

NIH3T3 cells. A month later, another report from the Herschman lab characterized the enigmatic effects of dexamethasone on COX with their ability to differentiate between COX-1 and -2 [56]. Herschman is generally credited with the discovery of COX-2 because his lab was the first to produce a genomic clone and publish almost all of the cDNA. They were also the first to use a COX-2 specific probe for Northern blots to demonstrate a time course and cell type specific expression.

In December of 1991, Donald Young's group published their results on the heels of Herschman's group with the complete sequence of the COX-2 [57]. Ryseck *et al.* from Bristol Myers Squibb in Princeton, NJ, published the most complete early characterization of COX-2 in a smaller journal six months later [58]. In their paper, they not only published the cDNA sequence, gene structure, time course of mRNA, and protein expression, but also its chromosomal location and initial data on the pharmacological characterization of COX-2 expressed in baculovirus. At that point in time the expression of proteins in baculovirus required an exceptional molecular biologist, a near clairvoyant microscopist[3], and a lot of time. Personal communications suggested that the Bravo R. group had the COX-2 protein cloned sequenced and expressed as early as 1989.

The anticipated pot-of-gold at the end of the rainbow awaiting a COX-2 selective inhibitor triggered a world class race to develop candidate drugs [28]. In 1999 Searle released Celebrex, the first FDA approved COX-2 selective inhibitor, and Merck followed closely with Vioxx. While the pharmaceutical companies were chasing the pot-

[3] Both the transformed virus and native virus appear identical, but because the coat protein is disrupted in the transformed virus, a decrease in refracted light can be observed. This optical property was used for the selection of positive clones in plaque lysis experiments. The slight difference was often very difficult to detect.

of-gold, the University of Rochester and Donald Young were waiting for it to arrive. In 1992, they filed a patent for the method of inhibiting COX-2. In April 2000, after Searle announced a blockbuster $1.5 billion in first year Celebrex sales, the University of Rochester announced that it had been awarded the patent. While the lawyers fight for the ownership of COX-2, the scientific community will probably always recognize the combined efforts of Herschman, Simmons, and Richards in their discovery of COX-2.

Transcriptional Regulation

Techniques used in transcriptional regulation studies. Several key types of experiments used to study transcriptional regulation include promoter activity assays, Electrophoretic Mobility Shift Assays (EMSAs), supershift EMSAs, and co-transfection.

In a promoter activity assay, a reporter gene is fused to a segment of DNA from the upstream region proximal to the transcriptional start site. Luciferase is a popular reporter gene because of its simplicity and sensitivity. Chloramphenacol acetyl transferase (CAT), and β-galactosidase are also used as reporters, but assays for their activity either require the use of radioactive substrates or are not very sensitive to lower promoter activities. A general strategy is to identify putative *cis*-acting elements via a database search, and then to design deletion constructs that progressively remove potential transcription factor binding sites. When the amount of activity drops significantly with the deletion of a region, that deleted region is considered relevant for the transcriptional activity of the gene. Once the minimal promoter is defined, mutation of the remaining consensus sequences can be used to narrow the search for essential *cis*-acting elements. With the target area more refined, EMSA experiments can be used to corroborate data from promoter activity analysis. EMSA experiments are used to locate specific regions of the promoter where proteins bind. Double stranded ^{32}P labeled DNA from relatively short regions of the promoter (20 to 200 bases) are incubated with extracts of nuclei prepared from cells in the induced and un-induced conditions. The samples are then separated on a non-denaturing acrylamide gel. If no protein binds the probe, then all of the DNA migrates though the gel. If binding does occur, then a shift in

the mobility of the probe is observed because the protein DNA complex moves much more slowly through the gel than the probe alone. In competitive EMSA experiments, a large piece of labeled DNA is competed with shorter pieces of DNA from the same region. The short unlabeled pieces are used in concentrations much higher than the larger labeled probe so that protein binding will be competed from the labeled probe resulting in the loss of the low mobility complex observed on the gel. Competitive EMSAs are also used to show that binding to a sequence is specific rather than non-specific.

Supershift EMSAs are experiments designed to identify specific proteins bound to a DNA probe. For example, the cAMP Response Element (CRE) consensus sequence can be bound by the CRE binding protein (CREB), several Activator Transcription Factors (ATFs) and by Activator Protein-1 (AP-1). In the COX-2 promoter there is a CRE that appears to be functional, so supershift EMSAs are performed with antibodies against CREB, ATF, and AP-1 proteins. If the mobility of the probe protein complex decreases (shifts up even further) or if the binding to the probe is blocked with one of the antibodies then that protein is likely to be bound to the DNA probe. The decrease in mobility is caused by the increase in the size of the complex bound to the probe. Alternatively, the antibody may interfere with DNA binding and block the interaction with the probe.

Co-transfection experiments are used to saturate the cell of interest with a protein to determine whether that specific protein will cause an effect on a promoter reporter plasmid. If a certain transcription factor is suspected to be involved in a response, its overexpression is expected to cause an increase in the transcription measured by a co-transfected promoter reporter construct. Dominant Negative (DN) experiments are used as a control. If co-transfection of a transcription factor upregulates promoter activity, then

co-transfection of a dominant negative version of the same transcription factor should downregulate the response. Dominant negative proteins are mutants that may lack a DNA binding domain, a functional activation domain (e.g. an essential serine that is phosphorylated is mutated to a non-phosphorylated amino acid), or *trans*-activation activity (may not be able to phosphorylate its target molecule.)

With these experiments one can define the active region of a promoter and use mutations to show loss of function to confirm the significance of a response element. Then, using EMSAs, one can show that the response element is bound specifically and, if possible, identify the protein in supershift experiments. If identification is possible, the co-transfection of that *trans*-activating factor should be expected either to cause increased promoter activity in the absence of stimuli or to greatly enhance the effect of a stimulus. Conversely, DN co-transfection should block stimulus induced promoter activation. By using upstream activators of the putative transcription factor and their DN counterparts, or specific inhibitors of signaling pathways, the general mechanism between stimuli and gene activation can be modeled. This is a basic strategy that has been employed in studying the promoter and transcriptional regulation of COX-2.

On a typical gene, the core promoter is located immediately upstream of the transcriptional start site. This core promoter region is where the general transcriptional machinery and RNA polymerase II bind to the promoter to initiate transcription at the correct location. In the upstream region adjacent to the core promoter is the regulatory promoter. This is where activating proteins bind to activate gene transcription. Enhancer regions are also present, but they can be located farther upstream or downstream of the core promoter. Activation of the gene occurs when the general transcription machinery

is recruited to the promoter by the activating factors in the regulatory promoter. In most respects, the COX-2 promoter is an ideal promoter to study. The core promoter contains a TATA box or TATA-like consensus sequence, and initial work has shown that the proximal region is capable of driving transcription of a reporter gene. (Figure 2) In contrast, the COX-1 promoter lacks a TATA sequence, and the upstream region provides only minimal activation of reporter constructs in fibroblasts [59]. In some extreme cases, the apolipoprotein B gene for example, the promoter lies more than 55 kilobases upstream from the coding region [60].

Investigations into the mechanism responsible for the expression of an induced gene need to consider several critical regulatory points. Regulation can occur at both transcriptional and post-transcriptional levels as well as at the level of translation. Before studying the transcriptional regulation of a gene, it is essential to determine the mode of regulation. DeWitt *et al.* showed that serum stimulation of fibroblasts results in a transient increase in the synthesis of a labile COX-2 mRNA that corresponds to a transient increase in protein expression and the previously observed increase in cyclooxygenase activity [61]. With the mode of regulation defined, studies of transcriptional initiation were logical to pursue.

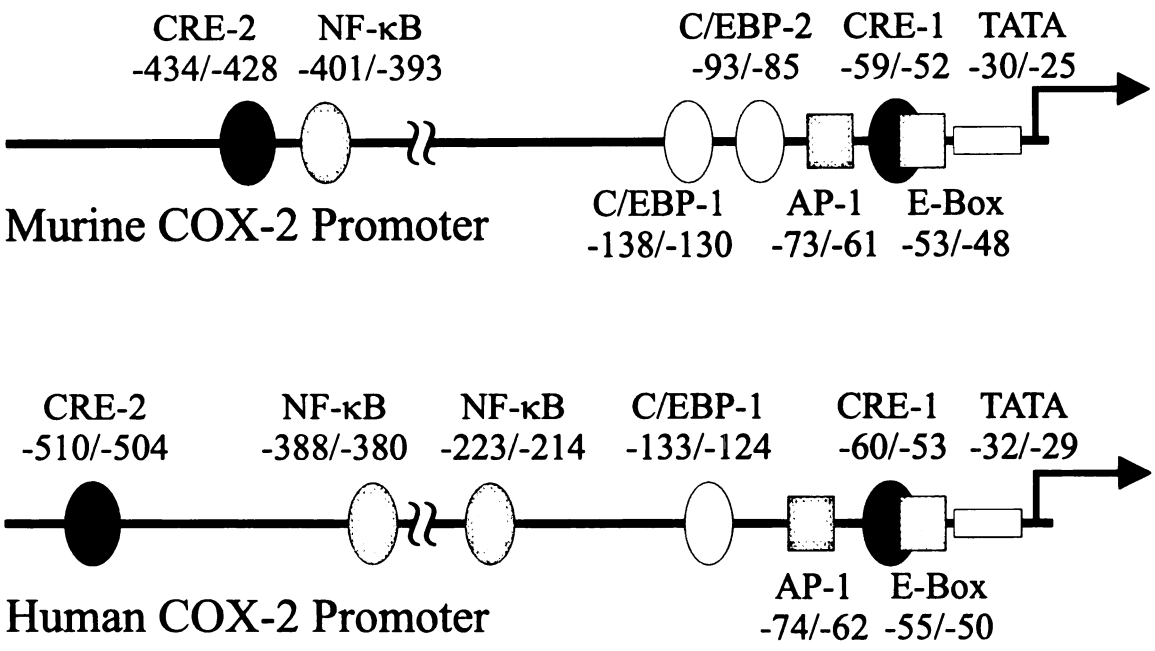


Figure 2. Response elements of the human and murine COX-2 promoters

Transcriptional Regulation of COX-2 in Fibroblasts

The first studies of COX-2 regulation were performed in NIH3T3 fibroblasts that were transfected with a temperature sensitive $pp60^{v\text{-}src}$ expression plasmid [62]. Promoter reporter constructs containing the region between –963/+70 or –371/+70 relative to the transcriptional start site, responded to both serum and TPA treatment when transfected into NIH3T3 cells [55]. The longer construct had only about 20% of the activity of the shorter construct. This was attributed to upstream negative regulatory elements. Similar experiments were also performed with the chicken COX-2 promoter in CAT reporter constructs in NIH3T3 cells, but the results were less dramatic and difficult to interpret clearly [63].

In the fibroblast model, the minimal promoter necessary for COX-2 activation by v-Src transformation involves only the first 80 bases of the promoter [62]. Deletion of the region from –80 to –40 resulted in a near complete loss of promoter activity. This region of the promoter contains a putative overlapping CRE and E-box. Overexpression of dominant negative CREB (no PKC phosphorylation site) or dominant negative myc (inactivated *trans*-activation domain) either blocked or significantly inhibited promoter activity, respectively. Mutation of the CRE blocked nearly all of the promoter activity, while the E-box mutation only blocked about half of the promoter activity. In EMSA and supershift assays, anti-CREB shifted a complex associated with the CRE and recombinant CREB bound to the EMSA probe. Methylation interference assays verified that there are protein DNA contacts throughout the region containing the overlapping CRE/E-box. Data from competitive EMSAs tentatively excluded the E-box as a participant in the response. Since v-Src is a protein tyrosine kinase that mimics the

activation of growth factor receptors resulting in ras mediated MAPK signaling, dominant negative ras was co-transfected with the promoter construct and v-Src. Dominant negative ras blocked nearly all the promoter activity, indicating that COX-2 induced expression is mediated by ras.

Xie *et al.* [62] mention that dominant negative CREB repressed promoter activity cannot be rescued by overexpression of CREB . Thus, CREB may not be the transcription factor mediating COX-2 induction by v-Src. An alternate hypothesis is that CREB is activated, but excess CREB may limit transcriptional activation by competing for a limiting cofactor necessary for interaction with the core transcription complex. Binding to the CRE/E-box response element was not inducible in cells grown in permissive and non-permissive temperatures for the expression of v-Src. This mechanism is consistent with a role for CREB. CREB can bind to DNA as a dimer but is inactive until it is phosphorylated at a critical serine residue (Ser133). Another interesting observation that was difficult to explain was that the region between –64 and –38, which contains the putative regulatory region, could not confer v-Src inducibility to a thymidine kinase promoter. This suggested that the CRE/E-box is necessary but not sufficient for activation.

One year later, a new v-Src mediated mechanism was proposed by Xie *et al.* who showed that both CREB and DN-CREB block transcription of a COX-2 reporter and concluded that CREB was not involved in the v-Src response [64]. Antibody to the Jun transcription factor could supershift the CRE/E-box probe and v-Src transformation potentiated Jun Kinase (JNK) activity. Based on evidence from promoter reporter co-transfections with different MAP kinases and dominant negative kinases, v-Src activation

of COX-2 was proposed to occur via two convergent pathways, 1) the "specific" pathway from ras/MEKK-1/JNKK (MKK4)/JNK leading to c-Jun phosphorylation, and 2) a pathway that was proposed to affect secondary response genes via raf-1/MAPKK/ERK1&2. This second pathway was hypothesized to result in the transcriptional activation of the c-Fos gene, which can form heterodimers with Jun resulting in more potent and stable activation of AP-1 transcription factors at the CRE site.

There is a curious inconsistency between the Jun transcription factor model and the previous CREB model. After being phosphorylated, Jun transcription factors bind to regulatory regions on promoters, yet binding to the CRE was not observed to be inducible. It is possible that Jun and CREB interact at two distinct sites that are very close together on the promoter and that both are required for transcriptional activation. Overexpression of functional CREB may compete for limiting amounts of cofactors necessary for its interaction with the transcriptional machinery, while overexpression of the dominant negative CREB may compete for binding sites. In either of these conditions, activation of the COX-2 promoter could be limited, so these experiments may not exclude CREB as a functional component of the transcription activating complex.

Early characterization of COX expression revealed that many different mitogenic and pro-inflammatory stimuli could cause an increase in both cyclooxygenase activity and mRNA levels. PDGF and serum (which contains undefined growth factors) were also shown to increase COX-2 promoter activity. In experiments similar to those used with the v-Src transformed fibroblasts, Xie *et al.* [65] demonstrated the necessity of the CRE element and presence of similar signaling pathways leading to COX-2 activation.

Collectively, these experiments formed the central dogma of COX-2 gene activation. COX-2 gene activation is primarily mediated by activation of the classical MAPK signaling pathway that terminates in the activation of AP-1 (Jun) through its interaction with a required CRE *cis*-acting element. However, more recent studies with human foreskin fibroblasts suggest that the process is much more complex [66]. These experiments indicate that salicylic acid inhibits transcriptional activation acting through a CAAT enhancer binding protein (C/EBP) response element. Additional experiments using IL-1β, TNF-α, or PMA as stimuli in promoter activity assays revealed that, in addition to the CRE-1, the C/EBP-1 and NF-κB sites are also required for transcriptional activation. Regulation beyond the level of transcription can also be more complex. In synovial fibroblasts, IL-1 stimulates the prolonged expression of COX-2 that is mediated by stabilization of mRNA, involving p38 activation [67].

Transcriptional Regulation of COX-2 in Epithelial cells

Cancer and prostaglandin production are linked through a mechanism that is still not clearly defined. It has been known for some time that people who take aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) have a 40% - 50% lower risk of colorectal cancer when compared with people who are not taking the drugs [68, 69] COX-2 inhibition significantly inhibits TPA-induced mouse skin tumor formation and azoxymethane induced rat colon tumorigenesis [70]. Aberrant expression of COX-2 has been observed in colorectal cancers, and in multiple cancers of the skin, head and neck, lung, breast, and stomach. COX-2 overexpression is associated with enhanced invasiveness and suppression of apoptosis [69, 71, 72] [73]. For these reasons, normal and transformed epithelial cells have been used to study COX-2 transcription.

Regulation of COX-2 transcription in epithelial cells shares some similarities with fibroblasts. Subbaramaiah *et al.* used transformed murine mammary epithelial cells to study the induction of COX-2 [74]. Transformation of murine mammary epithelial cells with ras or v-src prodigiously increased COX-2 mRNA and protein levels. To characterize the mechanism of transcriptional regulation, the authors used the promoter activity assays with 5' deletion constructs containing –40/+3, -80/+3, and –962/+3 of the murine COX-2 promoter. The authors' data showed that the long construct had approximately 7 to 10 times the promoter activity of the –80/+3 construct, yet the authors observed that "promoter activity was localized to a region between –80 and –40." Co-transfection with Jun enhanced promoter activity and dominant negative ras blocked induction, but to a lesser extent compared to the longest construct. This would tend to indicate that a larger portion of the promoter is necessary for the response, although there

is not a substantial loss in inducibility. While these results indicate that MAP kinase pathway can activate transcription from the minimal region of the COX-2 promoter, activation in epithelial cells is mediated by a more complicated mechanism.

Kim *et al.* set out to characterize the *cis*- and *trans*-activating factors in mouse skin squamous cell carcinoma JWF2 cells that constitutively express COX-2 [75]. Through a convincing set of experiments, they showed that the E-box and C/EBP sites act as positive regulatory elements, and binding sites for USFs and C/EBP transcription factors, respectively. In competitive EMSA assays, the overlapping E-box/CRE probe was competed by the same probe with a mutation in the CRE but not with a mutation in the E-box and general CRE consensus sequence did not compete binding for the native COX-2 E-box/CRE probe while a general consensus USF oligonucleotide did. In supershift assays, USF-1 and 2 antibodies shifted complexes and antibody to CREB, ATF-2, c-Jun, and c-Myc had no effect. Mutation of the C/EBP site dramatically reduced promoter activity and specific binding to the C/EBP probe was observed. C/EBP-δ and C/EBP-β were supershifted, and overexpression of C/EBP-δ doubled promoter activity. They also showed that C/EBP-δ mRNA is upregulated in JWF2 cells where it is not present in normal epithelial cells. To further test the involvement of C/EBP transcription factors, a dominant negative C/EBP transcription factor (CHOP-10) was co-transfected with the COX-2 promoter reporter. Overexpression reduced promoter activity in a dose dependent manner. Because mutations of either site bound by USF or C/EBP decreased promoter activity, the COX-2 promoter appears to require both sites for full promoter activity.

More recently, the ectopic expression of Wnt-1 has been linked to induced COX-2 in C57MG and RAC311 murine mammary epithelial cells [76]. Wnt-1 encodes a secreted protein that functions as a ligand for the Frizzled family of seven transmembrane receptors. Wnt1 signaling leads to the stabilization of cytoplasmic β-catenin, leading to the formation of β-catenin•TCF (T-cell factor) complexes and transcriptional activation. In cells expressing Wnt-1 COX-2 transcription, mRNA and protein levels are increased. Characterization the mechanism of Wnt-1 mediated COX-2 expression was done in a human embryonic kidney cell line to observe the effects of co-transfection with β-catenin and a handful of Ets transcription factors[76]. β-catenin stimulated only very weak COX-2 promoter activity, indicating that it acts on an intermediary factor rather than directly with the native promoter. Of the Ets transcription factors tested (Pea3, ER81, ERM, ETS-1, and ETS-2), co-transfection of Pea3 resulted in a high level of promoter activity. Pea3 expression is induced or at least present in Wnt-1 transformed cells and mammary tumors from Wnt-1 transgenic mice [76]. Characterization of *cis*-acting elements that respond to Pea3 was quite interesting. Deletion constructs of the human promoter from –1432, –327, and –220 all had similar levels of promoter activity. Deletion of the region between –124 and –220 resulted in a nearly complete loss of promoter activity. In experiments that were intended to exclude the NF-κB, C/EBP and CRE sites as possible candidates for Pea3 interaction, the authors were surprised to find that mutation of the C/EBP site completely eliminated promoter activity. The human COX-2 promoter has Pea3/Ets consensus sequences at –859 and –400, but not within the –220 to –124 region. However, there are several forward or reverse "GGAA/T" core Ets box sequences. Two possible mechanisms could account for the observation. Pea3 may actually bind to the C/EBP site and activate

transcription or, more likely, Pea3 binds to a C/EBP proximal site and functions in a synergistic fashion to activate transcription[76].

To test the model, the authors overexpressed C/EBP-α, β, and δ with the COX-2 promoter reporter and observed *trans*-activation with the C/EBP-α and δ isoforms, and decreased promoter activity with C/EBP-β[76]. In a second experiment, the authors also overexpressed Pea3 with DN-C/EBP (LIP) in increasing amounts and observed a dose dependent decrease in promoter activity. While the authors favored a model of Pea3 and C/EBP transcription factors working together from proximal sites because of the differences in C/EBP and Pea3 consensus binding sequences, their experiments do not discriminate between the two proposed modes. C/EBP could activate transcription independently of Pea3. The DN-C/EBP (LIP) has a functional DNA binding domain and a deleted *trans*-activation domain, so it probably competes with Pea3 for binding at a common site. Undoubtedly, this model will be explored further because of its potential utility in explaining the mechanism responsible for COX-2 expression in tumor types where inappropriate Wnt-1, β-catenin, and Ets transcription factor regulation are observed [76, 77].

COX-2 expression in epithelial cells is regulated in response to a number of tumor promoting compounds. Some of the more interesting compounds include benzo[a]pyrene and caffeic acid phenethyl ester in oral epithelial cells [78, 79], dihydroxy bile acids and curcumin in colonic epithelial cells [80, 81], and ceramide and resveratrol in mammary epithelial cells [82, 83]. Resveratrol is a phytoalexin found in grapes and their fermentation products, curcumin is responsible for the yellow color in turmeric, and caffeic acid phenethyl ester are all phenolic antioxidants that act like glucocorticoids to

downregulate COX-2 expression. Sphingomylenase, dihydroxy bile acids, and benzo[a]pyrene all up regulate COX-2 at least in part though their activation of MAPK signaling.

Retinoids suppress EGF induced COX-2 transcription and expression, as well as prostaglandin production, and promoter activity in oral squamous carcinoma cells [84]. The mechanism by which retinoids block COX-2 transcription is fairly interesting, because retinoids antagonize AP-1 mediated gene *trans*-activation. Since the COX-2 promoter lacks binding sites for the retinoic acid nuclear receptors in the proximal promoter region, it is unlikely that they interact directly with the COX-2 promoter.

If ligands for nuclear receptors are added to an appropriate system, the genes controlled by the receptor are expressed. At the same time, most genes controlled by AP-1 are downregulated. If AP-1 is reactivated, then the genes controlled by the nuclear receptor are downregulated [85]. Chris Glass's Cell paper "A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors" tested a hypothesis that the two systems competed for limiting amounts of CBP [86]. This competition provided a mechanism for mutual antagonism of these two classes of transcription regulators. This topic is the subject of a broad and intense field of research and has been reviewed by Chris Glass and others [85, 87-89].

With a basic understanding of this new and popular model of transcriptional co-activators competed between nuclear hormone receptors and AP-1 transcriptional activators, Dannenberg's lab set out to tie this mechanism to their previously observed results. They had observed that PMA mediates COX-2 induction through AP-1 *trans*-activation and that glucocorticoid and glucocorticoid-like compounds, as well as

PPAR ligands, had antagonized COX-2 induction. They showed that in PMA treated human epithelial cells, the PPAR-γ ligands, troglitazone, ciglitazone, and 15d-PGJ_2 all blocked COX-2 mRNA induction [90]. PMA induced PGE_2 production, and COX-2 protein levels were reduced to near background levels. In promoter activity assays, PMA induced activity was reduced to near background levels by the addition of either ciglitazone or 15d-PGJ_2. Co-expression of a DN-PPAR-γ, deficient in its ability to interact with CBP, or co-transfection of a decoy PPAR Response Element (PPRE) restored promoter activity in the presence of the PPAR-γ ligand. Both treatments inhibit *trans*-activation of PPAR responsive genes, resulting in an increase in free transcriptional co-activators. Co-transfection of the promoter reporter with NF-κB, CREB or C/EBP-α had no effect on ciglitazone blockade of PMA stimulated cells, but co-transfection of c-Jun or CBP each reduced the effect of the PPAR-γ ligand. Co-transfection of CBP and c-Jun together restored nearly all of the PMA induced activity. While it was already known that AP-1 participates in the activation of the COX-2 gene and that AP-1 mediated gene activation requires CBP, this paper showed for the first time that a transcriptional co-activator participates in the transcriptional activation of COX-2.

Transcriptional Regulation of COX-2 in Endothelial Cells

Jones *et al.* observed that PMA, IL-1β, TNF-α, and LPS induced COX-2 expression and prostaglandin synthesis in Human Umbilical Vein Endothelial Cells (HUVEC) [91]. Based on these observations, Inoue *et al.* investigated whether COX-1 or COX-2 was induced in Bovine Atrial Endothelial Cells (BAECs) [92]. While COX-1 was unaffected by the treatments, COX-2 mRNA was synergistically induced by LPS and TPA. They observed that the promoter activity induced from constructs containing –327/+59 or –1432/+59 of the human promoter were nearly identical. Their deletion constructs revealed that the NF-κB element (-223/-214) was necessary for full activity. Mutation of the C/EBP element resulted in a decrease in promoter activity, while the CRE-1 mutation had little effect, and a double mutation in both the C/EBP and NF-κB *cis*-acting elements reduced promoter activity to near background levels. These results were excitingly inconsistent with nearly all the previous observations. First, in fibroblasts the –1432/+59 construct had much less promoter activity than –327/+59 [55], indicating the presence of upstream repressors, but in epithelial cells, –1432/+59 had much more activity than –327/+59 [74], indicating that the authors clearly missed investigating an active upstream activator, but in BAEC [93] both constructs had the same activity indicating that –327/+59 actually contained the complete, necessary, regulatory region for induction in this system. Second, Mutation of CRE-1 usually had resulted in either significant decrease or ablation of promoter response, but here mutation of the CRE-1 resulted in a negligible decrease in promoter activity.

EMSA experiments in this system were even more unusual [92]. In BAEC nuclear extracts from unstimulated cells, very little binding to the CRE-1 probe was

observed; however, when the cells were transfected with C/EBP-α, β, or δ, significant specific binding was observed. In co-transfection experiments, C/EBP-δ induced COX-2 promoter activity in the absence of LPS, and mutation of CRE-1 blocked C/EBP-δ induced promoter activity, while mutation of the C/EBP response element only reduced promoter activity by about one-half. So, CRE-1 does not appear to be required for LPS and TPA induced COX-2 in BAEC, but it is required for C/EBP-δ dependent induction. It may be possible that an unusual C/EBP and CREB heterodimer, which has been observed with the human IL-1β promoter [94] in response to LPS, functions is some capacity in this system.

This LPS-stimulated BAEC model shows that in one cell type the CRE-1 site is necessary under some conditions and is unnecessary in others. In addition, it was the first clear example of the NF-κB site being necessary for activation of a COX-2 promoter reporter.

The NF-κB sites in the human COX-2 promoter were examined in HUVEC cells under hypoxic and normoxic conditions [95]. Hypoxia followed by reoxygenation activates NF-κB (p65/p50) in HeLa cells, [96] and COX-2 is induced under hypoxic conditions in HUVEC cells. Activation of COX-2 in this context appears to be mediated by a p65/p50 Rel protein dimer with the downstream NF-κB response element in the human promoter.

To quickly summarize the data discussed this far, fibroblasts really only need the first 80 bases of the promoter. Epithelial cells are a little more complicated and use mostly the CRE and C/EBP sites, perhaps in coordination (some say cooperatively) with each other, but may require more of the promoter for full activation. Endothelial cells,

which respond to a more diverse set of extracellular stimuli, use a combination of the NF-κB, C/EBP, and CRE sites, but none are absolutely required for at least partial activity. Transcription factors binding to the CRE include CREB/ATF and Jun family members, and maybe an undefined C/EBP heterodimer. The E-box (when necessary) is bound by USFs. The C/EBP response element is bound presumably but not definitively by several members of the C/EBP family. The NF-κB site is bound by a p65/p50 Rel dimer, and there may also be some undefined Pea3 binding sites that work in coordination with the C/EBP site. These transcription factors may be regulated by activation of PKC, JNK, p38, and ERK MAP kinase pathways. Pathways involved in NF-κB or Wnt-1 signaling can also *trans*-activate COX-2 transcription.

Transcriptional Regulation of COX-2 in Bone Tissue

The mechanism of bone resorption is cAMP dependent and appears to be mediated though the PGE_2 receptor subtype 2 and 4 (EP2 and EP4). In EP4 knockout mouse calvaria culture, bone resorption is heavily impaired relative to that of the EP1, EP2, and EP3 knockout mice. However, addition of an EP2 agonist in the $EP4^-/EP4^-$ tissue still stimulated bone resorption [97, 98].

COX-1 and 2 are both induced by similar stimuli in the MC3T3-E1 osteoblastic cell line. The induction of COX-1 at the mRNA level is only about 2 fold, and the increase in COX-1 protein expression is even less. In COX-2 knockout mice, bone development is similar to wild type mice; however, bone resorption is severely reduced. (PTH injected between long bones results in calcification of the joint in COX-2 knockout mice.) Therefore, COX-2 is thought to play a much more significant and active role in bone resorption and formation than COX-1[7, 16].

In calvaria and MC3T3-E1 cells, COX-2 expression is upregulated in response to treatment with basic Fibroblast Growth Factor (bFGF), Epidermal Growth Factor (EGF), Transforming Growth Factor (TGF-α and TGF-β), inteleukin-1 (IL-1), Tumor Necrosis Factor-α (TNF-α), Parathyroid Hormone (PTH), thrombin, bradykinin, forskolin, epinephrine, and prostaglandins PGI_2, PGE_2, $PGF_2\alpha$ or their stable analogues. [7, 16, 99].

The MC3T3-E1 cell line was isolated from mouse calvaria and is considered to be an osteoblast-like cell. Even before the discovery of an inducible COX isoform, EGF treatment of MC3T3-E1 cells had been shown to induce the release of prostaglandin metabolites, the most predominant of which was PGE_2 and was referred to as "bone resorption factor." The release of arachidonate metabolites was dependent on both

transcription and expression of a new prostaglandin synthase activity and mimicked the results observed in tissue preparations [100].

After the identification of the second COX enzyme, Pilbeam *et al.* [101] examined COX-2 expression in the MC3T3-E1 cell line and found that treatment of serum starved cells with serum, TGF-β, PMA, forskolin, or PGE_2 resulted in a transient increase in COX-2 mRNA accumulation and protein expression. Because of the rapid and potent changes they observed, they suggested that COX-2 is involved in bone responses to acute stress, such as mechanical strain, inflammation, and injury. TNF-α causes bone resorption *in vivo* and *in vitro*. MC3T3-E1 cells respond to TNF-α by rapidly expressing COX-2 and by releasing PGE_2 as well as lesser amounts of other arachidonic acid metabolites [102-105]. At the transcriptional level, COX-2 mRNA is synthesized rapidly and transiently with mRNA levels falling to near baseline by 3 hr. Similar results are seen with serum treatment as well, following a time course similar to that observed in fibroblasts [101]. However, in the TNF-α response, COX-2 mRNA levels increase again between 6 and 12 hr to maximal levels that are maintained for more than 24 hr. The post-6 hr burst is reduced by the cyclooxygenase inhibitor NS398, leading to the hypothesis that this delayed response is in part due to a positive autocrine feedback loop [102].

Yamamoto *et al.* [102] transfected 5'serial deletions of a 621 bp promoter construct into MC3T3-E1 cells that were treated for 12 hr with TNF-α. Promoter analysis showed that the NF-κB and C/EBP sites were necessary for full promoter activation because mutation or deletion of the NF-κB or C/EBP sites potently reduced promoter activity. In EMSA experiments, inducible binding to the NF-κB and C/EBP probes was observed in nuclear extracts from MC3T3-E1 cells treated with TNF-α for 1 hr. Anti-p50

and anti-p65 supershifted the bound NF-κB probe, and anti-NF-IL6 (C/EBP-β) supershifted the C/EBP probe. The authors [102] suggested that COX-2 transcription may be regulated similarly to the IL-6 and IL-8 genes, where cooperative binding of C/EBP and NF-κB transcription factors to those promoters was observed [106].

The temporal regulation of COX-2 transcription in response to TNF-α in MC3T3-E1 cells occurs in several phases [102]. First, there is a rapid and transient induction between 0 and 2 hr followed by an intermediate repression or loss of mRNA accumulation between 2 and 6 hr Finally, there is a late phase of induction where there is a slower increase in mRNA starting at about 6 hr post treatment, which reaches a maximum level by about 12 hr. It is possible that the upstream and downstream regions of the promoter mediate different phases of the response, but since luciferase activity from the promoter reporter was only measured at 12 hr several events are confounded. If partial loss of promoter activity is observed as a result of a mutated response element, it is impossible to determine if the mutation affected activity during the early or delayed phase or during both phases of reporter gene activation. When bimodal responses are observed, mechanistic studies of transcription factor binding should also be done during each phase to check for consistency of the response across the time course of activation.

Wadleigh *et al.* [107] examined the effects of serum, bFGF, PDGF, PGE_2 and TNF-α+IL1-β treatment on MC3T3-E1 cells. After 4 hr of each treatment, COX-2 mRNA as well as luciferase activity levels from a promoter reporter (–724/+7) were increased. Response element mutation constructs were tested in MC3T3-E1 cells treated for 4 hr with bFGF. This study identified and characterized a new degenerate C/EBP site (C/EBP-2) located at –93/-85. Mutation of C/EBP-2 had little effect, while mutation of

C/EBP-1 reduced promoter activity by about 25%, and a double mutant decreased promoter activity by more than 50%. Mutation of the CRE-1 site eliminated promoter activity, and a mutation in the NF-κB site and the E-box had no significant effect. Since the CRE-1 mutant and the double C/EBP mutant had the biggest effects on bFGF stimulation, the authors tested these two mutant constructs against serum, bFGF, PDGF, PGE2, and TNF-α+IL-1-β and observed nearly identical decreases in promoter activity as was observed with bFGF. Because MAP kinase signaling pathways mediate COX-2 induction in other cell types in response to mitogenic and inflammatory stimuli, the authors co-transfected DN-MEKK and DN-JNK with the wild type promoter and found that half of the promoter activity was abolished. Co-transfections with c-Jun, C/EBP-β, and C/EBP-δ superinduced reporter activity, and co-transfection with CREB and DN-C/EBP-β (LIP) blocked the induced promoter activity. The authors [107]concluded that bFGF, PDGF, PGE_2, and TNF-α +IL-1-β activate transcription from the CRE-1 site via a mechanism that involves c-Jun and the MEKK/JNK signaling pathway as well as transcriptional activation at the C/EBP sites in response to C/EBP transcription factor family members.

Okada *et al.* [108] identified an AP-1 consensus site at –69/-63 in the murine promoter. This site in the COX-2 promoter is one base off the canonical consensus sequence, but in this case, a close match seems to be good enough. PMA stimulated promoter activity in MC3T3-E1 cells was increased by about 5 fold in both –963/+70 and –371/+70 reporter constructs indicating that the region upstream of –371, which includes the NF-κB and CRE-2 sites, is not necessary for PMA stimulated activation of COX-2. The authors prepared single and double mutations of the CRE-1 and

AP-1 sites in the –371 construct to be transfected into MC3T3-E1 cells treated with either PMA or serum for 3 hr. While the AP-1 and CRE-1 single mutants had different effects with either PMA or serum treatment, in both cases the double mutant had significantly less promoter activity than either of the single mutants. EMSA experiments performed in this paper yielded very interesting results. The AP-1 site probe was bound specifically and inducibly by nuclear proteins from cells treated with either PMA or serum and antibody to either c-Jun or c-Fos blocked binding of the probe, while the CRE-1 probe was bound constitutively by extracts from both the PMA and serum treated cells. Since this AP-1 site is conserved in the murine, rat, bovine, equine, and human promoters, the authors suggested that a physiological role for the AP-1 site may exist. This relationship between two proximal response elements, where both are necessary for a full activation but inducible binding is only observed at one site, is highly suggestive of a cooperative interaction between the DNA bound transcription factors and transcription complex.

Mechanical loading deforms the extracellular matrix producing fluid flow in the osteocyte lacunar-canalicular network that results in elevated COX-2 expression and production of PGE_2, and $PGF_2\alpha$ [101, 109]. *In vivo* studies indicate that COX-2 selective inhibitors can block mechanical stress induced bone formation [101]. Loss of mechanical stress due to prolonged immobilization or exposure to microgravity (space flight) results in loss of bone density [101, 109]. In addition, *in vivo* long-term administration of exogenous prostaglandins increases bone formation [7].

Fluid sheer stress rapidly induces prostaglandin production through activation of a cytoskeleton-associated calcium channel that results in the activation of PKC. To study the effects of fluid sheer force, Ogasawara *et al.* [109] cultured MC3T3-E1 cells on glass

slides that were placed in a parallel flow path chamber. In these experiments, the flow rate of media was kept constant while the gap between the slide and the top of the chamber was decreased. This increased the velocity of the media across the slide, which increased the sheer stress on the cells. The authors found a "dose" dependent relationship between sheer stress and COX-2 expression. By RT-PCR, the authors observed a sustained increase in COX-2 mRNA by 3 hr with only a small increase observed at 1 hr. 5' serial deletions from –959 were prepared, and the authors observed that deletion of the region containing the C/EBP site nearly eliminated all the steer stress induced response. Mutations in the C/EBP-1, AP-1, and CRE-1 sites all reduced promoter activity by more than 50%, while mutations at the CRE-2, NF-κB, C/EBP-2, and E-box had no effect. The authors also performed double and triple mutation analysis of the C/EBP-1, AP-1, and CRE-1 sites, all of which resulted in promoter activity that was lower than the unstimulated wild type promoter construct. The observations from a comprehensive and compelling set of EMSA experiments produced results that were very interesting. Nuclear extracts were prepared from control cells and cells stimulated with sheer stress for 1 or 3 hr. They observed inducible binding to the C/EBP-1 probe that was supershifted with antibody to C/EBP-β. Binding to the AP-1 probe was also inducible and was supershifted by anti-c-Jun/AP-1 antibody. Binding to the CRE-1 probe was constitutive, and it was supershifted by antibody to CREB at 1 and 3 hr and by also anti-phospho-CREB after 3 hr.

Because of the signaling pathways that are activated by sheer stress, one might expect to find similarities within the transcriptional mechanism observed in response to PMA treatment. Indeed, the findings of Okada *et al.* [108]with PMA treated MC3T3-E1

cells are consistent with those of Ogasawara *et al.* in sheer stressed stimulated MC3T3-E1 cells. Okada *et al.* [108]compared the effects of the CRE-1 and AP-1 mutations in serum and PMA treated MC3T3-E1 cells. They found that the CRE-1 site was more important for the serum response and the AP-1 site was more important for the PMA response, but with either treatment. The double mutation significantly lowered promoter activity relative to the single mutants. This finding is relatively consistent with the observations of serum and mitogen stimulated MC3T3-E1 cells where the CRE-1 mutation eliminated promoter activity [102, 107].

Perhaps these data indicate that gene activation requires both the AP-1 and CRE-1 sites. If one site is removed, then most of the *cis*-activation potential is lost, and if both are removed, then nearly all of the gene activation is removed. In these studies there is an important incongruent detail that is overlooked. In all of these studies, binding to the CRE-1 site is constitutive. This is not consistent with mechanism of AP-1 transcription factors. The AP-1 transcription factors are phosphorylated when MAPK signaling pathways are activated, leading to inducible, transient binding to target sites on gene promoters. I think it may be possible that the AP-1 site which is immediately upstream of CRE-1 site is where the inducible binding of AP-1 transcription factors occurs, and that occupation of the overlapping CRE-1 and E-box sites is necessary for *trans*-activation. If this is true then removal or deletion of the of the CRE-1 site would block *trans*-activation, as has been observed, and removal or deletion of the AP-1 site would also block *trans*-activation. This is probably a key part in the puzzle to understanding the transcriptional activation of the COX-2 gene, which will be discussed in more detail later.

As a slight tangent to the work discussed above, glucocorticoids have been shown to be potent inhibitors of inflammatory mediators and the stimulated release of prostaglandins. In addition, glucocorticoids have been found to decrease bone resorption. Two recent papers should have very real impacts on the national space program and the diets of astronauts. The glucocorticoid-like natural product, humulon (((R-)-3,5,6-trihydroxy-4,6-bis(3-methyl-2-butenyl)-2-(3-methyl-1-ox-obutyl)-2,4-cyclohexadien-1-1), which is isolated from hops extract, was found to inhibit bone resorption (IC_{50} 5.9 nM) and COX-2 activity (IC_{50} 1μM) [110]. Another glucocorticoid-like natural product, resveratrol, a phytoalexin found in grapes, blocked the transcriptional activation of COX-2 by interfering with the activation of PKC, ERK, and c-Jun [83, 111]. Together, these data suggest that Tang® should be replaced with other more beneficial beverages on longer space flight missions where bone resorption critically impacts human performance and wellness.

Transcriptional Regulation of COX-2 in Granulosa Cells

Prostaglandins are necessary mediators of the reproductive process. COX-2 deficient female mice are infertile because of problems in ovulation, fertilization, implantation, and decidualization [112]. Functional promoter studies in gonadatropin stimulated pre-ovulatory rat granulosa cells showed that a transiently transfected -2698/+23 promoter reporter construct mimicked the *in vivo* induction kinetics of the COX-2 enzyme. From a set of 5' serial deletion reporter constructs, they found the region between –194 and -54 was critical for reporter activation. The region upstream of -194 contained possible negative regulatory elements, which was also observed by Fletcher *et al.*[55]. EMSA experiments helped to identify a region between -192 and -110 that exhibited significant specific binding. This region of the rat promoter contains an AP-1 site at -165/-159, as well as a C/EBP site at -142/-120 [113]. The C/EBP site was bound by the transcription factor C/EBP-β but not C/EBP-α or C/EBP-δ in EMSA supershift assays. *In vivo* immunoblot and northern analysis confirmed that C/EBP-β was constitutively expressed in hCG stimulated pre-ovulatory follicles. When the C/EBP consensus was mutated in a -192/+23 reporter construct, inducibility in response to forskolin, FSH, and LH was greatly reduced. Although the organization of the rat promoter is different from the murine and human promoters, similar *cis*-acting elements mediate COX-2 gene activation by their interactions with similar *trans*-activating factors. Interestingly, similar results were observed with the bovine promoter in bovine granulosa cells. Promoter analysis and EMSA experiments showed that the C/EBP but not the CRE-1 site was necessary, and that the C/EBP site was only bound by C/EBP-β [114].

When different mechanisms of gene transcription are observed across different cell types and in response to different stimuli, it becomes evident that one specific signaling pathway is not used to activate the COX-2 gene. Even within one cell type, different stimuli can activate transcription of COX-2 by activating different pathways of cellular signaling. The most convenient, reduced model for studying COX-2 transcription has been the fibroblast stimulated with MAPK activating mitogens; however, even in this cell type there are exceptions when more or less of the promoter is required to drive promoter reporter activity.

Lipopolysaccharide Signaling

The LPS Molecule. Bacterial lipopolysaccharide is a complex molecule with three covalently linked domains. (Figure 3) The O-antigen polymer is an oligosaccharide with up to 40 repeated units. This domain is highly immunogenic and varies greatly between bacterial strains [115]. The core region is a phosphorylated non-repeating oligosaccharide that is required for the outer membrane of bacteria to function against antibiotics. The third domain is the lipid A, which functions as the hydrophobic anchor for LPS in the outer membrane. The lipid A domain consists of a pair of phosphorylated and acylated β,1-6-linked glucosamine molecules. Variations in the acyl chains determine the biological activity of the LPS. The six 3-hydroxy fatty acids of the lipid A are generally saturated and are between 14 and 18 carbons long[116]. Deacylated LPS no longer retains its inflammatory properties and becomes an antagonist for active LPS [117]. Likewise the heavily modified LPS of *Rhodobacter sphaeroides*, with its unusually short acyl chains, functions as an antagonist for the action of *E. coli* endotoxin [116].

The LPS Receptor. LPS is a membrane forming amphiphile that is relatively insoluble in aqueous solutions and diffuses slowly from membranes and aggregates [118]. In serum free buffers, mg/ml concentrations are required to generate a cellular response. With serum, ng/ml concentrations of LPS are sufficient to elicit a response because of the presence of the 60 kDa LPS Binding Protein (LBP) . LBP forms high affinity complexes with the lipid A moiety of LPS and in turn; can interact with both soluble and membrane bound monocyte differentiation antigen CD14 [119]. LPS can be transferred from LBP to CD14, which is involved in enhanced sensitivity to LPS and in the clearance of LPS by mediating its transfer to lipoprotein particles or phospholipid

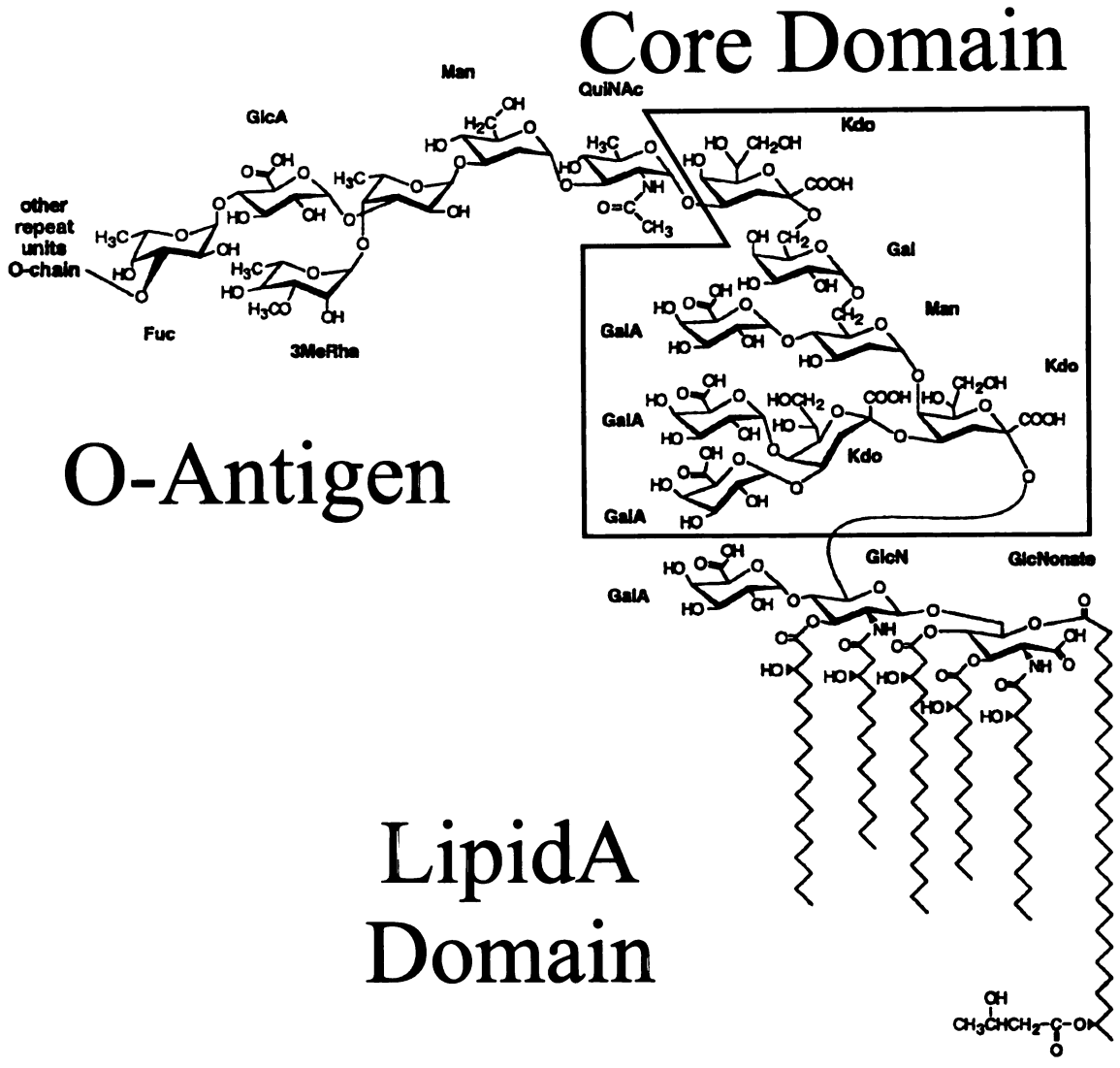


Figure 3. Lipopolysaccharide Molecule A representative structure of the core region, part of the O-antigen chain, and lipid-A domain of lipopolysaccharide.

Abbreviations: GalA, galacturonic acid; GlcA, glucuronic acid; Kdo, 3-deoxy-D-manno-2-octulosonic acid; GlcN-onate, 2-amino-2-deoxygluconic acid; QuiNAc, 2-N-acetamido-2,6-dideoxyglucose (N-acetylquinovosamine); GlcN, glucosamine; Man, mannose; 3MeRha,3-O-methylrhamnose; Fuc, fucose.

Forsberg LS, Carlson RW. 1998. The structures of the lipopolysaccharides from *Rhizobium etli* strains CE358 and CE359. The complete structure of the core region of *R. etli* lipopolysaccharides. J. Biol. Chem. 273:2747–57

vesicles [120]. The clearance of LPS is a separate event from LPS induced signal transduction [118, 120-122]. CD14 is attached to the extra cellular membrane by a glycosyl phosphatidylinositol anchor and has no transmembrane domain. It is unable therefore, to transfer a signal through the cell membrane. However, CD14 is an LPS receptor, and blockade of CD14 with antibody blocks LPS induced TNF-α, IL-1β, IL-6, and IL-8 release as well as endotoxin induced shock in whole animal studies [123]. On the other hand, the LPS hypo-responsive mouse, C3H/HeJ, expresses normal amounts of CD14 on cell surfaces suggesting that additional factors are required for an LPS response [124].

In the drosophillia world of development, a receptor involved in dorsal ventral patterning was discovered with an intracellular carboxyl terminal domain that is similar to the carboxyl terminal of the IL-1 receptor. This receptor, named Toll, participates in a signaling cascade leading to the activation of DIF and Dorsal, the drosophillia homologues of an NF-κB like signaling pathway, which is essential for the anti-fungal immune response in flies[124].

Several mammalian homologues of Toll have been identified and are referred to as Toll-Like Receptors (TLR). The intracellular domain of TLRs are similar to the intracellular portion of the IL-1 receptor, which suggests that the mammalian homologues may participate in signaling pathways similar to the IL-1 receptor. The TLR proteins were first implicated as potential LPS receptors when positional cloning of the gene responsible for the LPS hypo-responsive phenotype of the C3H/HeJ mice was mapped to the TLR4 gene [124]. In addition, the LPS insensitive mouse line C57/10ScCr is null for the TLR4 locus and does not express any TLR4 cDNA [124]. Both the TLR2 and TLR4

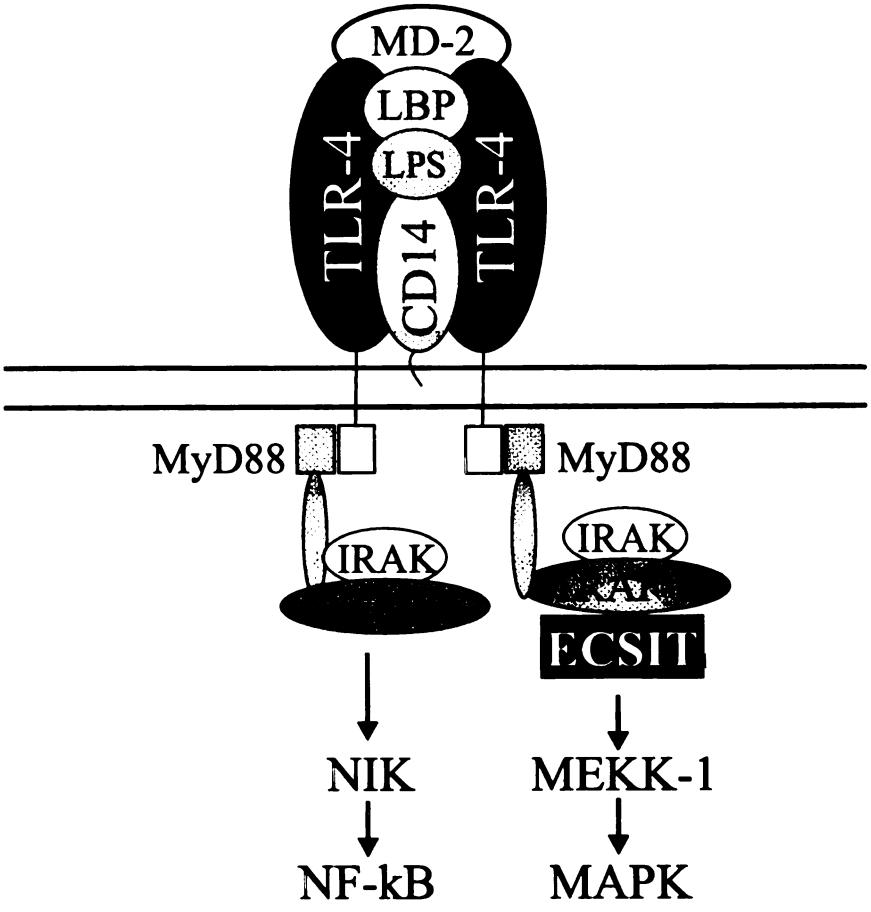


Figure 4. The TLR4 Receptor Complex

receptor mRNAs are highly expressed in peripheral blood leukocytes, monocyte, and macrophage populations. Transfection of either receptor confers LPS responsiveness to LPS insensitive cells, and co-transfection of CD14 further enhances the response [125, 126]. However, highly purified LPS does not signal through TLR2, and TLR2 gene knockout mice remain sensitive to LPS where as disruption of the TLR4 gene results in a loss of LPS responsiveness [127]. The TLR2 receptor appears to be involved in a more broad recognition of other bacterial cell wall components such as bacterial peptidoglycan from gram positive cells and bacterial lipoproteins [128, 129]. The Toll receptors appear to function as cell sensors of microbes. So far, ten TLRs have been identified. In addition to TLR2 and TLR4, TLR6 is thought to be involved in recognition of bacterial lipoproteins, and in cooperation with TLR2 and TLR9 recognizes bacterial CpG DNA.

The molecular interactions leading to LPS signaling involve a complex assembly of proteins on the extracellular surface of the cell (Figure 4). LPS is first bound by the amino-terminal domain of LBP, and then the carboxyl terminal domain of LBP associates with CD14. LPS triggers the physical association of CD14 and TLR4 and then upon binding ligand, the TLR dimerizes [130]. The TLR4 complex also requires the cell surface protein MD-2, which also appears to be necessary for signaling through TLR2. Although its exact function is still unknown, MD-2 is thought to play a role in the stabilization of the receptor complex [131, 132].

Intracellular signaling is mediated by interactions with the intracellular Toll/IL-1R (TIR) domain. Signaling through IL-1R, TLR2, and TLR4 is mediated by the interaction of the receptor TIR domain, with the TIR domain of myeloid differentiation factor (MyD88), which facilitates the assembly of the toll receptor complex. This

complex includes the IL-1R associated kinase (IRAK), TNF receptor associated kinase-6 (TRAF-6), and the evolutionary conserved intermediate in toll (ECSIT) signaling factor [124].

Within 5 to 15 min the activated TLR4 receptor complex activates a broad range of signaling molecules and the release of second messengers. Rapid activation of p38, ERK1/2, and JNK activities are observed; I-kBα and I-kBβ are degraded; phospholipase A_2 (PLA_2), phospholipase C (PLC), and sphingomylenase (SMase) activities are upregulated; Protein Kinase C α and ξ (PKC) and protein kinase A (PKA) are activated; and members of the Src family of tyrosine kinases are activated as well. [133-140]. In macrophage and monocyte cells, these more immediate events are then followed by autocrine effects mediated by the release of TNF-α, IL-1β, and prostaglandins.

MAPK Signaling Induced By LPS. LPS induced signaling can be divided into two general types, MAPK signaling and NF-κB signaling[4]. Within the MAPK category, three main nodes are activated: the Jun N-term Kinase (JNK), the Extracellular Receptor Kinase (ERK), and p38 (Figure 5). The TLR4 receptor signaling complex initiates the activation of these kinases. This complex includes the recently discovered ECSIT, which cleaves inactive MEKK-1 (MAP3K) to its active form [141]. (Figure 6) MEKK-1 best known for activating MKK4/SAPK [142], which activates JNK resulting in phosphorylation and activation of AP-1 transcription factors (specifically Jun family members) [143]. MEKK-1 can also activate MKK3/6, which results in activation of p38 [142, 144, 145]. MKK4 is also activated and is able to activate p38 in models of inflammation. [146, 147]. Additionally, MEKK-1 has been found to activate IKK

[4] Caveats of Signaling Experiments, Appendix A

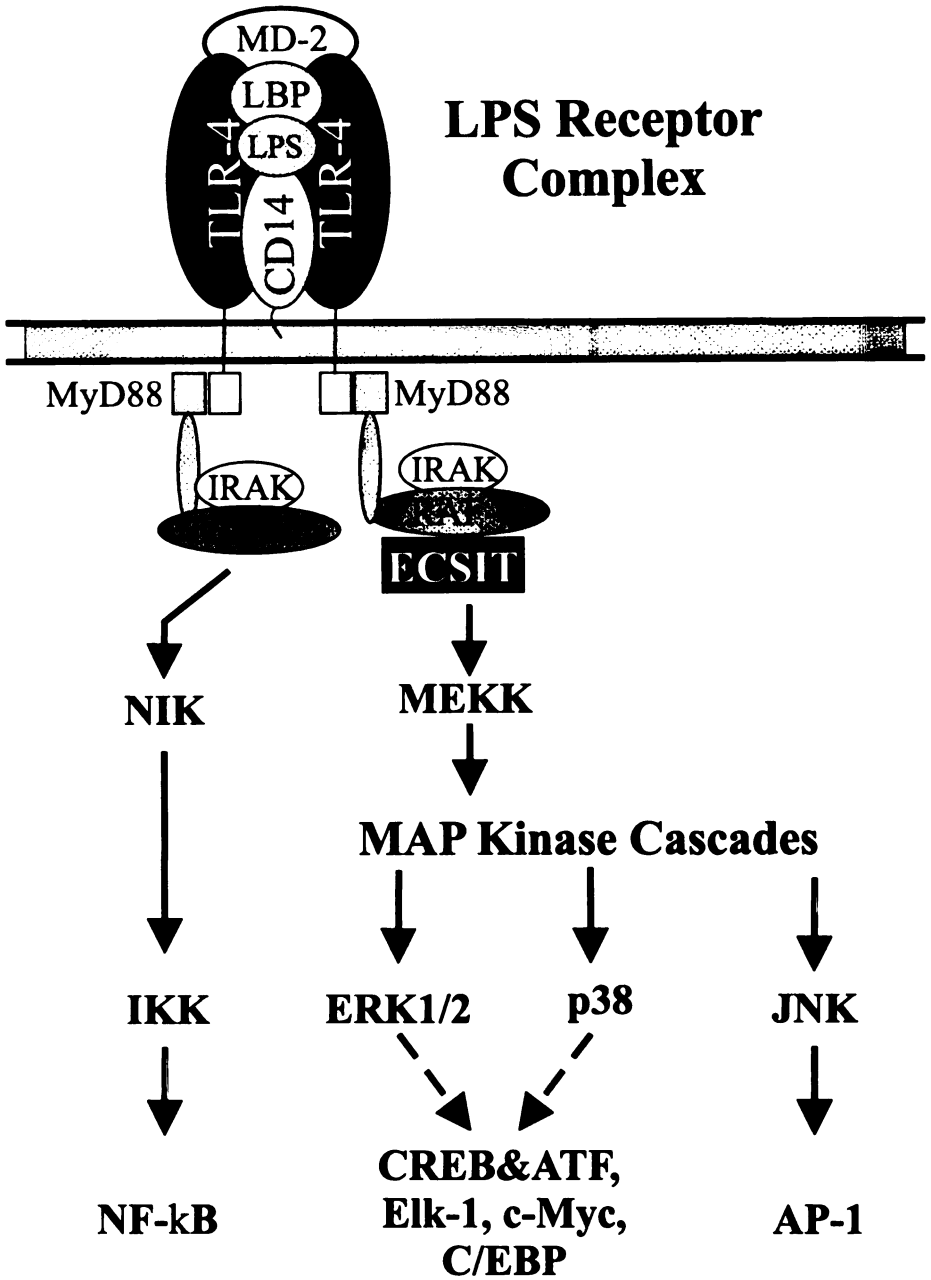
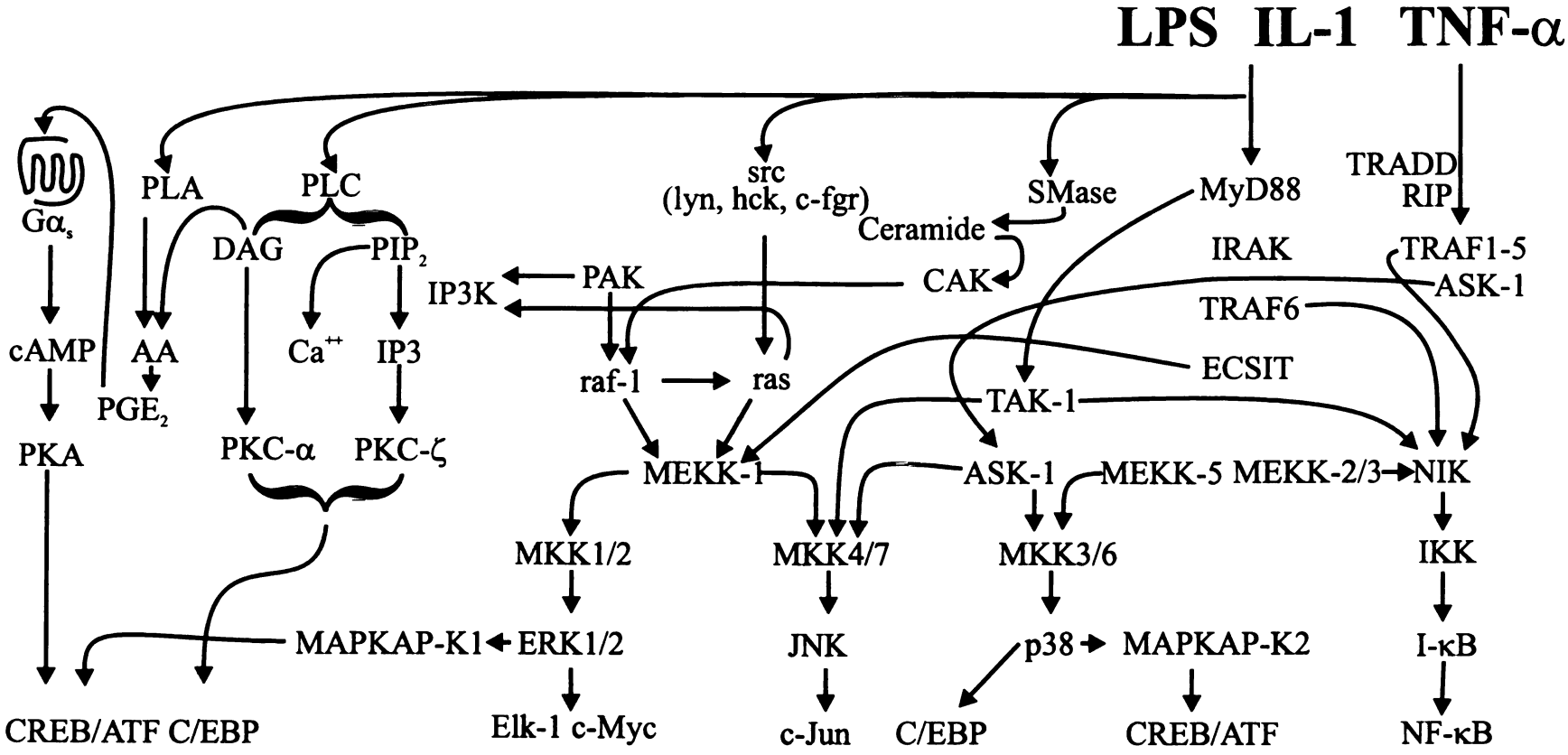


Figure 5. General signaling pathways activated by LPS

Figure 6. Detailed signaling pathway map: A schematic representation of the signaling pathways activated directly or indirectly by LPS. Key to abbreviations: AA, Arachidonic Acid; ASK-1, apoptosis signal-regulating kinase; ATF, Activator of Transcription Factor; C/EBP, CAAT Enhancer Binding Protein; CAK, Ceramide Activated Kinase; CAMP, Cyclic Adenosine Monophosphate; CREB, cAMP Response Element Binding Protein; ECSIT, Evolutionary conserved intermediate in toll; Gas, Stimulatory G-protein Subunit; IKK, Inhibitor of kB Kinase; IP3, phosphatidyl-inositol-3; IP3K, phosphatidyl-inositol-3-kinase; IRAK, Interleukin-1 Receptor Associated Kinase; JNK, c-Jun N-term Kinase; LPS, Lipopolysaccharide; MEKK, Mitogen Activated Protein Kinase Kinase Kinase; MKK, Mitogen Activated Protein Kinase Kinase; MyD88, Myocyte Differentiation Factor; NIK, Nuclear Factor-kappa B Inducing Kinase; PAK, p21 Associated Kinase; PIP2, Phosphatidylinositol-4,5-bisphosphate; PKA, Protein Kinase A (cAMP); PKC, Protein Kinase C (Calcium); PLA, Phospoholipase A; PLC, Phospoholipase C; RIP, [TNF] Receptor Interacting Protein; TAK-1, Transforming growth factor-beta Activated Kinase-1; TNF-a, Tumor Necrosis Factor-a; TRADD, Tumor Necrosis Factor Receptor Associated Death Domain; TRAF, Tumor Necrosis Factor Receptor Associated Factor.


LPS
IL-1
TNF-α
Gα$_s$
PLA
PLC
src
(lyn, hck, c-fgr)
SMase
MyD88
TRADD
RIP
DAG
PIP$_2$
Ceramide
IRAK
TRAF1-5
IP3K
PAK
CAK
ASK-1
TRAF6
cAMP
AA
Ca^{++}
IP3
ECSIT
raf-1
ras
TAK-1
PGE$_2$
PKA
PKC-α
PKC-ζ
MEKK-1
ASK-1
MEKK-5
MEKK-2/3
NIK
MKK1/2
MKK4/7
MKK3/6
IKK
MAPKAP-K1
ERK1/2
JNK
p38
MAPKAP-K2
I-κB
CREB/ATF C/EBP
Elk-1 c-Myc
c-Jun
C/EBP
CREB/ATF
NF-κB

leading to NF-κB activation [140, 148]. LPS causes the stimulation of a variety of tyrosine kinase activities, and inhibition of tyrosine kinase activity with inhibitors such as herbimycin blocks the LPS response in RAW 264.7 cells [136]. Several Src family members are activated by LPS and may contribute to the tyrosine phosphorylation of ras/raf-1 [149-151], which are involved downstream of MKK1/ERK1/2. Alternatively, LPS activates p21 associated kinases (PAKs) that mediate the activation of ras, raf-1, and rac1. Specifically, PAK1 is activated by TRAF2/6 and is required for efficient NF-κB activation and transcription of the TNF-α gene in LPS-stimulated RAW 264.7 cells [152].

The mechanisms directly responsible for the LPS-induced increases in PLC, PLA, and SMase are unknown, but as a result of the activation of these lipases, a number of lipid mediators are formed. PLC generates both phosphoinisotides and diacylglycerol, which cause the release of intracellular calcium from the endoplasmic reticulum into the cytoplasm and activation of protein kinase C (PKC) respectively [153, 154]. PKC-α and PKC-βII are calcium dependent PKC isoforms activated in response to LPS [153, 154]. The IP3K dependent isoform, PKC-ξ, is activated and probably plays a more direct role in the activation of the COX-2 gene [155]). In response to LPS, IP3K activity is observed. This kinase is most likely activated by either ras or PAK [156, 157]. SMase causes the release of ceramide, which results in the activation of Ceramide Activated Kinase (CAK) and followed by the activation JNK and AP-1. The lipid A portion of LPS is structurally similar to ceramide and may also activate CAK directly, but since LPS treatment causes the release of ceramide it is not clear if this mechanism is important in the RAW 264.7 cell model [133, 134].

NF-κB Signaling Induced by LPS. There are five mammalian members of the NF-κB transcription factor family, c-Rel, Rel-A/p65, Rel-B, p105, which is cleaved to the active p50, and p100, which is cleaved to the active p52 form. NF-κB transcription factors exist as dimers and are sequestered in the cytoplasm by an Inhibitor of κB (I-κB), which is thought to mask the nuclear localization signal of the transcription factor. Both p50 and p52 can form homodimers and are thought to act as negative regulators, while Rel-A/p65 and Rel-B primarily form heterodimers and function in the *trans*-activation of NF-κB responsive genes [158]. When I-κB is phosphorylated, it quickly becomes ubiquinated, and is degraded by the proteosome, allowing unmasked NF-κB transcription factors to enter the nucleus. The activating phosphorylation event is catalyzed by IKK (I-κB kinase). The IKK complex is composed of IKKα and IKKβ, both of which can phosphorylate I-κB, and a scaffolding protein, IKKγ, that is necessary for activity [158]. IKK is phosphorylated to its active state by the NF-κB Inducing Kinase (NIK). NIK is activated by TRAF-2/6 and *in vitro*, TRAF-1/3/5 can activate NIK. TRAF-6 is a component of the TLR4 receptor complex and is activated through its interaction with IRAK and MyD88 on the cytoplasmic domain of the liganded Toll-like receptor. Overexpression of MEKK-1/2/3, PKC-ξ, COT/Tpl-2, and TAK-1 also results in the activation of IKK, indicating that many different signaling networks could result in NF-κB activation. Since NIK is a MAP kinase kinase kinase (MAP3K) it is not completely surprising that the –SXXXS– motif targeted by NIK, on IKK is also phosphorylated by other MAP3Ks like MEKK-1/2/3, COT kinase, and TAK-1 [158-161] [162]].

Autocrine Amplification of LPS Induced Signaling. In addition to the initial activation of LPS inducible signal transduction pathways that activate COX-2

transcription, autocrine activation of other signaling pathways also occurs. Two very potent mediators of inflammation are IL-1 and TNF-α, and both are released from RAW 264.7 cells in response to LPS. IL-1R signals through a mechanism that is thought to be very similar to that of the TLR4 [124]. In the cascade of events activated by TNF-α, one additional kinase, ASK-1, is activated through its interaction with TRAF2. ASK-1 activates MKK4 leading to JNK activation [163, 164]. PKA activation is observed as a result of increased cAMP levels, due to the activation of adenylate cyclase by $G\alpha_s$ from activated prostaglandin receptors [139]. Cytokines such as IL-6 and IL-8 are also released, and IL-6 participates in the activation of C/EBP transcription factors. Although C/EBPs are activated in the initial LPS response, autocrine activation may further potentiate their activity [139, 165].

Resolution of the LPS Response. Downregulation of the LPS response is complex and gene specific. Activation of p38, JNK, and ERK in RAW 264.7 cells occurs between 10 and 30 min after LPS treatment, and the activity of JNK and the ERKs is returned to near baseline levels by 2 hr, while p38 remains elevated for at least 24 hr but at levels much lower than the initial peak of activity [143, 166, 167] . I-κB degradation is rapid and the protein is typically resynthesized, which results in the resolution of the NF-κB response within 1 to 2 hr. Adenylate cyclase is activated as a result of LPS simulation. The increased levels of cAMP downregulate some genes as do synthetic cAMP analogues. If cAMP levels are sufficiently high the activation of NF-κB and the release of TNF-α are prevented [168]. The time course of TNF-α production peaks near 8 hr and then returns to near base line by12 hr. When cAMP production is inhibited, the time course of expression is extended [169]. Early studies with LPS-and interferon-induced

macrophage and monocytes showed that exogenous prostaglandin and dibutyryl cAMP could negatively regulate tumoricidal function (TNF-α release and expression)[170-175]. COX-2 expression seems to be at least partly enhanced by cAMP, but cAMP alone is not sufficient to induce COX-2 expression [166]. Prostaglandins produced as a consequence of enhanced COX-2 expression may also work to downregulate the response by producing 15-deoxy-$\Delta^{12,14}$-PGJ_2, a prostaglandin metabolite that can interfere with NF-κB activation both at the level of I-κB phosphorylation and at the level of transcription by a PPAR-γ [140, 148].

Transient Activation and Persistent Responses. Considering the time courses of the pathways induced by LPS, it is curious that COX-2 expression remains elevated while most of the other genes induced by LPS are downregulated. In other cell systems where COX-2 transcription has been studied, the gene is rapidly and transiently activated; however, when the NF-κB site is involved in the transcriptional activation of the promoter, then COX-2 seems to be induced for longer periods of time. Questions exist about whether persistent activation of COX-2 is tied to NF-κB signaling, and if so, what is the mechanism for this effect?

Initial NF-κB transcription factor activation is dependent on the phosphorylation and degradation of IκB. After IκB is degraded, NF-κB dimers enter the nucleus and bind DNA. At the same time, newly synthesized un-phosphorylated IκB enters the nucleus and binds NF-κB. This binding event masks the Nuclear Localization Signal (NLS) of the transcription factor and destabilizes its interaction with DNA. This general paradigm of NF-κB activation has a few exceptions. There are several members of the IκB family that modify the NF-κB activation event. The term, "IκB", nearly always refers to the IκB-α

family member, which acts as described above. The second major isoform, IκB-β, functions in an entirely different capacity [176]. IκB-β is also rapidly degraded [140] upon activation of IKK; however, the newly synthesized un-phosphorylated IκB-β does not destabilize NF-κB DNA binding or mask the NLS. As a result, NF-κB can remain active in the nucleus bound to a promoter, protected from IκB-α by its occupation by IκB-β, in the absence of prolonged IKK activation. Thompson *et al.* suggested that the persistent response involving IκB-β may occur in of chronic inflammation, infection, stress, or differentiation [176-178]. This second isoform of IκB may play a role in the LPS-induced signal transduction in RAW 264.7 cells. Both IκB-α and IκB-β are present and degraded upon LPS treatment [140]. Another IκB family member, Bcl3, is involved in unusual NF-κB interactions . Bcl-3 is located in the nucleus where it interacts preferentially with p50 and p52 NF-κB family members [179]. Bcl-3 is thought to bind to p50 and p52 homo and heterodimers to facilitate their removal from DNA, thereby allowing RelA/p65 and RelB containing NF-κB transcription factors to bind and activate gene transcription. Alternatively, there are cases where Bcl-3 is involved in gene *trans*-activation through its interaction with p50 homodimers and the transcriptional co-activators SRC-1 and CBP [179-181]. The mechanism underlying the prolonged LPS induced COX-2 response has not yet been studied and may be caused by a complex interaction of activated transcription factors; however, persistent activation of NF-κB is likely to be involved [106, 182].

Transcriptional Regulation of COX-2 in the Macrophage

In the late 1970s and early 1980s peripheral and peritoneal macrophage cells were shown to have inducible prostaglandin production and cyclooxygenase activity [183-185]. The murine macrophage cell lines RAW 264.7, J477, P388D1, and PU-5-15 all metabolized arachidonic acid to prostaglandin products in response to phagocytic stimuli, endotoxin, and calcium ionophore [186]. Of these cell lines, RAW 264.7, has been used extensively as a model for macrophage activation and the inflammatory response. RAW 264.7 cells were cloned from Abelson leukemia virus transformed murine peritoneal macrophage cells and have functional characteristics of a macrophage cell. In addition, treatment with phorbol esters is not required to sensitize the cells to LPS or other activating stimuli [187].

The inducible cyclooxygenase activity presented an intriguing problem. LPS treated RAW 264.7 cells quickly begin to release arachidonic acid via a stimulated PLA_2 activity resulting in arachidonic acid release and prostaglandin production [188]. However, if exogenous arachidonic acid was added to the macrophage cells in the absence of treatment it was also metabolized to prostaglandins by a constitutive cyclooxygenase activity. The constitutive cyclooxygenase activity was relatively low compared to the inducible, delayed phase of cyclooxygenase activity. This delayed phase was dose and time dependent and was blocked almost completely by the glucocorticoid dexamethasone (Dex). But Dex had little, if any, effect on the lower levels of constitutive cyclooxygenase activity. Interestingly, while enzyme activity was "prodigiously" induced, protein levels observed on immuno blots and by immunoprecipitation was only increased by 2 to 3 fold. This led to speculation about a constitutively expressed

cyclooxygenase activity and an inflammation inducible cyclooxygenase activity [42]. As soon as the second isoform of prostaglandin synthase was discovered (COX-2), its increased mRNA levels were quickly demonstrated to be inducible in response to LPS in primary macrophage cells and cell lines [189-192]. In addition, PMA, TNF-α, and IFN-γ were also observed to induce the expression of COX-2 protein and mRNA in murine and human primary monocytes and macrophage cells and cell lines [193-195].

The first step in studying gene regulation is to identify the mode of regulation, followed by identifying and defining the promoter and then identifying *cis*-acting DNA elements and the relevant *trans*-acting factors. A number of experiments in human, rabbit, rat, and mouse cells and cell lines show that LPS increases COX-2 mRNA, protein, and enzyme activity levels roughly proportionally [189, 193-196]. These experiments indicate that COX-2 is regulated at the level of transcription, although there are some exceptions involving post-transcriptional regulation.

After determining the mode of regulation, the next step should be to identify the region of DNA that is involved in the regulation of the gene. The conclusions from the data derived from the transcriptional regulation studies in other cell types and stimuli have been applied to macrophage cells. The studies of COX-2 gene regulation in LPS treated macrophage cells assume that the response elements that are necessary in other systems are also necessary in macrophage cells.

The first studies of COX-2 gene regulation in macrophage cells used human U937 cells [197]. U937 cells treated with TPA or PMA differentiate from a pro-monocyte to an adherent, monocytic stage. During differentiation to a macrophage like cell, low levels of COX-2 are expressed, and inflammatory mediators such as LPS TNF-α, and IL-1 can

further induce the expression of COX-2. In the undifferentiated state, the U937 cells do not express COX-2 mRNA or protein and treatment with inflammatory mediators does not activate the COX-2 gene.

After TPA-induced differentiation, nuclear protein bound CRE-1 in EMSA experiments, and mutation of CRE-1 drastically reduced basal activity from a human COX-2 promoter reporter [197]. LPS treatment of differentiated cells induced COX-2 expression and caused binding of a p65/p50 NF-κB transcription factor to the human downstream NF-κB response element. The NF-κB transcription factors are known to be involved in the activation of many LPS inducible genes. So, to test whether or not the NF-κB response element contributed individually to the LPS response, the authors prepared a human promoter construct (-327/+29) with mutations in both CRE-1 and the C/EBP response elements. Stable transfection of this construct into differentiated U937 cells confers LPS inducible promoter reporter gene activity [198]. This experiment suggests that the NF-κB site contributes to the LPS response. This experiment is highly cited as the demonstration that the NF-κB response element is required for LPS-induced COX-2 activity, but there is a potential problem with this experiment. Stable transfection of a promoter reporter is subject to positional effects, and many genes are activated by LPS so their increase in promoter activity may be a complete artifact. Second, and perhaps more importantly, the experiment assumes that only the CRE-1, C/EBP-1, and NF-κB sites are involved in the activation of the COX-2 promoter, and this has not been verified.

The observations that CRE-1 is bound by protein in nuclear extracts only after phorbol ester treatment of U937 cells and that mutation of CRE-1 does not eliminate

LPS-induced COX-2 promoter activity are interesting. In a related study, U937 cells were treated with platelet microparticles and transfected with a hCOX-2 promoter construct (–87/+123) containing CRE-1 but not upstream elements. Little if any basal promoter activity was seen with these constructs and it was not inducible [199]. In contrast, a long construct (-1840/+123) was potently induced by the platelet microparticles. The transcription factors, c-Jun and Elk-1, were co-transfected with the large promoter construct with and without the platelet microparticle treatment. Alone, the transcription factors did not cause an increase in promoter activity; however, after platelet microparticle treatment, large increases in the hCOX-2 promoter activity were observed. Platelet microparticles activate PI 3-kinase resulting in the transient activation of several PKC isoforms, ERK1/2, JNK, and p38, and probably represent a more physiological priming of the U937 cells than stimulation with PMA. These experiments are important because they show that the pre-primed promoter is not sensitive to Jun and Elk transcription factors, indicating that these factors are insufficient to induce transcription of COX-2 independent of other endogenous factors. The experiments also show that in macrophage like cells a larger portion of the promoter is necessary for a COX-2 response, indicating that *trans*-activation of the COX-2 gene is more complex than in fibroblast cells.

A cell line that is somewhat similar to the U937 cell line is the human THP-1 pro-monocytic cell line. THP-1 cells have also been used to study the transcriptional regulation of COX-2. J Mestre *et al.* [155] examined the effects of the CRE-1, C/EBP, and NF-κB sites in the LPS response in three hCOX-2 promoter reporter experiments. First, they used 5' deletion constructs starting with –327/+59, which contained all three

response elements. Deletion of the region containing the downstream NF-κB site had no effect, while deletion of the C/EBP containing region resulted in about a 50% decrease in basal and induced promoter activity; further deletion of the CRE-1 containing region abolished both basal and induced promoter activity. In the second experiment, the authors used promoter constructs with single mutations in the CRE-1, C/EBP, and NF-κB sites. Only the CRE-1 mutation resulted in a decrease in promoter activity. In the third experiment, the authors used double mutations in the CRE-1, C/EBP, or NF-κB sites to determine if promoter activity could be activated by one response element alone. The intact NF-κB site had very little promoter activity, and the intact C/EBP and CRE-1 constructs had less than one-third the wild type promoter activity. These data indicate that none of the single major response elements are sufficient to activate the reporter gene. In addition, this also shows that the CRE-1 site is necessary for full promoter activation but it not absolutely required (mutation of the CRE-1 site does not ablate promoter activity), and that the downstream NF-κB site has little impact on gene activation. Parallel experiments with these hCOX-2 constructs were performed in RAW 264.7 cells with similar results.

Dominant negative ERK-2, p38, JNK, and PKC-ξ were co-transfected with a human COX-2 promoter reporter (–327/+59). Each of these kinases can superinduce LPS stimulated levels of promoter activity, but none of the dominant negative kinases abrogated promoter activity. The authors conclude that redundancy in the signaling pathways leading to the activation of the COX-2 gene prevented any single dominant negative kinase from affecting promoter activity. Using the single intact response element constructs, the authors co-transfected the ERK-2, p38, JNK and PKC-ξ kinases to see

which sites on the promoter are acted on by each signaling pathway. The intact CRE-1 site was not activated by p38, while the intact C/EBP site construct was activated by p38 as well as PKC-ξ but not JNK or ERK-2, and all the kinases activated the construct with the intact NF-κB site. (Activation in this context indicates that the treatment caused about a two-fold increase in promoter activity compared with the co-transfection with an empty vector.) These data indicate a mechanism with multiple redundancies can function minimally on two of the three response elements participates in the LPS induced activation of the COX-2 gene. LPS activates a broad range of signaling pathways and autocrine signaling loops resulting in multiple mechanisms for activating each signaling pathway. It is therefore not completely surprising that the COX-2 gene is activated thorough a redundant mechanism by LPS.

Wadleigh *et al.* [200] set out to show that a specific signaling pathway is primarily responsible for the LPS induced COX-2 response in RAW 264.7 cells. Dominant negative kinases of the MAPK pathway were co-transfected with a mouse COX-2 promoter reporter containing –724/+7, which includes the all the major upstream response elements. Dominant negative MEKK1 and JNK reduced promoter activity by about 50%, and overexpression of c-Jun superinduced promoter activity. Both dominant negative ras and raf-1 had no effect on promoter activity, which is in contrast to what is observed with the TNF-α promoter where either dominant negative ras or raf-1 block activation. The recently identified ECSIT protein is downstream of the TLR4 LPS receptor complex and is capable of activating MEKK1. Dominant negative ECSIT blocked about 50% of the COX-2 promoter activity. Dominant negative ERK1 and ERK2 only decreased promoter activity by about 20%, and overexpression of CREB reduced

LPS induced promoter activity. Several co-transfection experiments with C/EBP transcription factors were used to identify their involvement in the LPS induced COX-2 promoter activity. They found that dominant negative C/EBP-β (LIP) blocked the promoter activation while overexpression of C/EBP-β resulted in much higher levels of LPS induced activity. C/EBP-δ was also used and resulted in superinduced basal and induced promoter activity levels.

The NF-κB signaling pathway was not observed to be involved in the activation of the COX-2 promoter construct [200]. A mutant Iκ-B plasmid was co-transfected with a COX-2 promoter reporter and a reporter with four repeats of the classical NF-κB response element. The dominant negative Iκ-B had little effect on COX-2 promoter activity but blocked LPS stimulated activation of the NF-κB reporter construct.

The results of the signaling experiments were confirmed with a promoter analysis study. Using the –724/+7 murine COX-2 promoter reporter construct, the authors observed that mutation of CRE-1 completely eliminated promoter activity, and mutation of C/EBP-1 reduced the promoter activity to about one-third of the wild type promoter. Mutations in the C/EBP-2 and NF-κB response elements did not significantly impact the LPS induced promoter activity.

The authors conclude that the CRE-1 site is the most important site for LPS-induced COX-2 activation and that ras/raf-1 independent activation of c-Jun via a ECSIT/MEKK1/JNK signaling pathway is required for COX-2 induction. Optimal induction also requires the C/EBP site and activation of one of the C/EBP transcription factor family members, and NF-κB activation is not required for COX-2 activation in RAW 264.7 cells.

Proof of NF-κB. So far we have observed the downstream human NF-κB response element is not absolutely necessary for LPS-induced transcription, so it may be that the upstream NF-κB element is responsible for the LPS-mediated response.

The human COX-2 promoter contains two NF-κB response elements. The position and sequence of the upstream site is similar to that of other animals, but the downstream site is not conserved and appears to be the result of a 15-20 bp insertion that is not present in COX-2 promoters from other species. The two sites have been compared in two different studies 1) in vascular endothelial cells under hypoxic conditions and 2) in bronchial epithelial cells stimulated with IL-1. In response to hypoxia, the downstream site plays as more significant role as a binding site for p60/p50, but in response to IL-1 the upstream site is bound with higher affinity by p65/p50 and p50/p50 than is the downstream site [201, 202]. In the studies discussed above, the human promoter constructs that were used had the upstream NF-κB site deleted, and in this context, mutation of the downstream site has little effect on promoter activity. This indicates that the downstream NF-κB site may not have a significant effect in promoter activation. It may be that the human COX-2 promoter has two functional NF-κB sites; however, the upstream site is highly conserved in other species, while the downstream site does not exist. The down-stream NF-κB response element may add to the functional activation of the COX-2 promoter, but it is likely that this lower affinity binding site functions as an enhancer of the upstream NF-κB binding site.

The data from Wadleigh *et al.* [107] demonstrate that NF-κB activation is independent of COX-2 activation in LPS treated RAW 264.7 cells. Is there any reason to believe that NF-κB activation is linked to the activation of COX-2? Several studies have

tied COX-2 transcription and expression to NF-κB activation. In human rheumatoid synovial fibroblast cells and human messangial cells, IL-1β upregulates COX-2. Decoy NF-κB oligos transfected into the cells decrease COX-2 activation, and expression of antisense p50 or p65 also decreased the level of COX-2 mRNA and protein [202, 203]. Both the NF-κB elements of the human COX-2 promoter are bound by p50/p65 in IL-1β stimulated human bronchial epithelial cells, and co-transfection of the human promoter reporter with p50 or p65 enhances induced promoter activity, although in TNF-α treated MC3T3-E1 cells, overexpression of p50 does not affect promoter activity [102, 201]. In the murine J774 macrophage cell line, LPS induced the expression and mRNA levels of COX-2. Treatment with proteosome inhibitors prevent the degradation of IκB resulting in substantially reduced levels of NF-κB activation. These drugs also inhibit increases in COX-2 transcription and expression [204, 205]. Similar results have also been observed in TNF-α treated fibrosarcoma cells [206].

In contrast to the observations of Wadleigh *et al.*[200], there are several reports that the NF-κB signaling pathway is essential for the activation of COX-2 in LPS induced RAW 264.7 cells [207, 208]. A series of co-transfection experiments with a murine COX-2 promoter reporter and components of the NF-κB signaling pathway provide compelling evidence [208]. Starting at the level of the LPS receptor, activated TLR4 receptor activates COX-2 promoter activity, while signaling deficient DN-TLR4 blocks COX-2 promoter activation. The effect of the dominant negative TLR4 is overcome by the addition of constitutively active MyD88. Overexpression of NIK and IKK-β superinduced LPS stimulated promoter activity, while dominant negative NIK blocked the LPS effect. Dominant negative Iκ-B, similar but not identical to what was used by

Wadleigh *et al.*, blocked activation of the COX-2 promoter. In TNF-α stimulated colonic epithelial cells, overexpression of dominant negative I-κB blocks COX-2 expression and prostaglandin production [209]. In all the co-transfection experiments by Rhee et al, a multiple NF-κB response element containing reporter plasmid was used as a control, and similar responses were observed with both this control and the COX-2 promoter reporter construct. [190, 208]. The results from these papers show that NF-κB activation is required for maximal expression of COX-2 in LPS stimulated RAW 264.7 cells.

CHAPTER 2

UPSTREAM NF-κB AND cAMP RESPONSE ELEMENTS IN CYCLOOXYGENASE-2 GENE EXPRESSION IN LPS-STIMULATED MACROPHAGE CELLS

Summary

Cyclooxygenase-2 (COX-2) is rapidly induced in RAW264.7 murine macrophages in response to lipopolysaccharide (LPS). *Cis*-acting elements of the murine proximal COX-2 promoter include an overlapping cAMP response element (CRE-1) and E-box at -59/-48 and two C/EBP response elements located at -138/-130 and -93/-85. The promoter also contains an NF-κB site at -401/-393 that has been proposed to be a target of LPS-activated Rel/NF-κB transcription factors. Using promoter deletion and mutational analyses, we have determined that the NF-κB response element is required for maximal promoter activity in LPS-treated RAW 264.7 cells. The NF-κB site is bound by p65/p50 heterodimer after 1 hr of lipopolysaccharide stimulation, but after 12 hr the NF-κB site is bound by a p50 homodimer. The p50 homodimer is typically associated with negative regulation of inflammation-related genes. However, the results of nuclear run-on assays indicate that elevated rates of COX-2 transcription occurred at the time when the NF-κB site was occupied by the p50 homodimer. We have also demonstrated that a conserved cAMP response element (CRE-2) located at -434/-428 in the murine promoter is necessary for maximal LPS-induced COX-2 promoter activity. This newly defined CRE-2 is bound by a CREB/ATF transcription factor and the CREB binding protein (CBP) in nuclear extracts prepared from RAW 264.7 cells. Curiously, the binding of these factors is similar in extracts from both control and LPS-treated cells. Pair wise

comparisons of the effects of mutations at the CRE-2 site and the other previously identified response elements indicate that the CRE-2 and NF-κB sites may function together in the *trans*-activation of the COX-2 gene.

Introduction

Prostaglandin endoperoxide H synthases, commonly known as "cyclooxygenases," catalyze the committed step in the metabolism of arachidonic acid to prostaglandins [9]. There are two isoforms of COX, the predominantly constitutive isoform, COX-1, and an inducible isoform, COX-2. Although both enzymes catalyze the same reaction with similar kinetics, studies with isoform specific inhibitors and COX-1 and COX-2 knock out mice suggest that there are some physiological processes that require one specific enzyme and others where both isoforms function together [8]. COX-2 expression is upregulated by a variety of stimuli in different cell types [9]. Bacterial lipopolysaccharide (LPS) is a potent inducer of COX-2 activity in macrophage cells and mediates signaling through the toll-like receptor-4 (TLR4), which results in the activation of JNK, ERK, p38, NIK, and PKC signaling pathways [161]. LPS-induced COX-2 transcription is regulated through multiple redundant mechanisms involving several central response elements present in the COX-2 promoter [155]. The CRE at –57/-52 is necessary for mediating the effects of a wide variety of stimuli, while a pair of C/EBP sites 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 contain NF-κB and cAMP response element pairs located between 400 and 550 bp upstream of the transcription start site (Figure 7). Here we show that the conserved NF-κB site at –401/-393 and the upstream CRE (CRE-2) located at -434/-428 in the murine promoter are required for maximal induction of COX-2 by LPS in RAW 264.7 cells. We also have identified nuclear proteins associated with these response elements.

The NF-κB site is necessary for induced COX-2 promoter activity in TNF-α stimulated MC3T3-E1 cells [102] but has not been demonstrated previously as being important for the LPS response in macrophage cells. Recently, however, Rhee *et al.* [208] demonstrated that blocking NF-κB activation at several levels results in a large decrease in COX-2 promoter activity. The CRE-2 site has only previously been tested by Ogasawara *et al.* [109] 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.

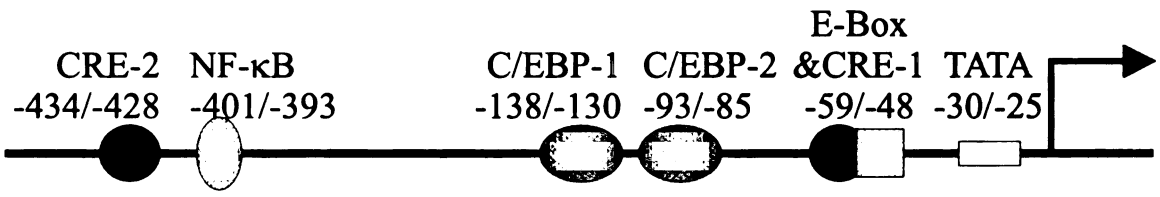


Figure 7. Schematic representation of the murine COX-2 promoter.

Materials and Methods

Reagents and Antibodies. Lipopolysaccharide (LPS) from *Salmonella minnesota* was purchased from Sigma. Antibodies to CBP, CREB, p65 and p50 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibody to phospho-CREB was purchased from Upstate Biotechnology (Lake Placid, NY). Complete™ Protease Inhibitor Cocktail and Pefabloc were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Poly(dI-dC)-Poly(dI-dC) average length 8517 bp[5] 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 (Rockford, IL).

Plasmids. The various regions of the mCOX-2 promoter were cloned into pGL3basic (Promega). The -966/+23 construct was cloned from a KpnI and HindIII fragment. The constructs containing -459/+23 and -414/+23 were cloned by PCR amplification with 3'-XhoI and 5'-HindIII tailed primers. Constructs containing -350/+23, -170/+23, and -98/+23 were cloned into the SmaI/HindIII sites of pGL3. 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. Mutations are summarized in Table III. pRC-p50 expression plasmid was provided by Richard Schwartz (Michigan State University), and the mCOX-2 plasmid, pSVLN-muCOX-2, was provided by David DeWitt (Michigan State University).

[5] The length of this product varies from lot to lot and is very different between manufacturers. This batch seemed to be better at blocking non-specific binding and reducing the background than other preparations that had shorter lengths.

Cell Culture and Transfections. RAW 264.7 cells (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% 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 5×10^5 cells/ml one day prior to transfection. A luciferase reporter plasmid (2.5 μg) and a pCMV-β-galactosidase plasmid (Pharmacia) 0.5 μg 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 (200ng/ml) stimulation for 12 hr. 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 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.

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 (10m 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 ug/ml)) and hybridized with a probe in TES/NaCl Solution (10 mM Tris 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 pSVLNmuCOX-2 and a 1.3 kb EcoR1, XhoI fragment of a β-Actin 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 Assay. 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 (Roche) and 1X Compete Protease Inhibitor Cocktail (Roche). Oligonucleotide probes (Michigan State University Macro Molecular Structure Facility) were annealed in T4 PNK Buffer and then end-labeled with T4 Poly Nucleotide Kinase (New England Biological) and γ^{32}P-ATP (New England Nuclear). The sequences of the probes are summarized in Table III. The probes were electophoresed 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 $MgCl_2$, 1mM EDTA and 0.1% SDS, ethanol precipitated and resuspended in TE. Binding reactions were performed with nuclear extract (5 μg of protein) and probe (ca. $2x10^6$ dpm) in the presence of 100 mM KCl, 20 mM HEPES pH 7.9, 1mM EDTA, 10% Glycerol, 2mM Pefabloc, 1X Complete Protease Inhibitor Cocktail, 2 mM DTT and 2μg of Poly(dI-dC)-Poly(dI-dC) and electrophoresed on 5% TAE acrylamide gels at 150 VDC for 1.5 hr. For EMSAs, the probe was incubated with the nuclear extracts for 1 hr at 25°C, and for the supershift assays, the nuclear extracts were combined with the probe 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, 12 hr. The cells were rinsed twice with ice cold PBS and were scraped into 1 ml of PBS. The cells were centrifuged briefly and resuspended in 1 ml lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% IGEPAL CA-650 (Sigma)) for 5 min. The nuclei were pelleted 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 the run-on reaction, 225 ul of 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 RNase-OUT (Life Technologies), and 100 µCi α-^{32}P-UTP. After 15 min at 37°C, 1 ml 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 upper aqueous phase was removed and combined with 500 µl 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 dH_2O. The RNA was further purified and 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 1M 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-Actin plasmid (Stratagene), empty vector, or vehicle without plasmid. After baking, the membranes were pre-hybridized in 5x SSC, 50% formamide, 5x Denhardt's, 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).

Results

Upstream response elements in LPS-induced COX-2 gene expression. Deletion constructs of the murine COX-2 promoter were inserted into a luciferase reporter plasmid and used to transfect RAW264.7 cells. Transfected cells were treated for 12 hr with or without LPS to determine which region of the COX-2 promoter are necessary for maximal LPS responses (Figure 8). The data are presented are from three independent experiments that were preformed in triplicate. The measurement of relative light intensity from the luciferase activity (or Relative Luciferase Units, RLU) was normalized to the units of β-galactosidase expressed from a separate pCMV-βgal plasmid. The height of each bar represents the average fold increase in promoter activity between control and LPS stimulated RAW 264.7 cells for each promoter reporter plasmid. The error bar represents one standard deviation relative to the average fold increase in promoter activity observed across the three independent experiments. Deletion of the region from –459 to –414 removes a conserved CRE element (CRE-2) without eliminating the adjoining, conserved NF-κB response element. When the CRE-2 region was removed, more than half of the LPS-induced COX-2 response was eliminated. Subsequent deletions that removed the NF-κB and C/EBP-1 response elements caused no further decreases in the LPS-induced promoter responses. Because the focus of our study was to examine the roles of the more distal response elements, additional deletion analyses (e.g. of the C/EBP-2 element or the CRE-1) were not performed. These latter two *cis*-acting elements have been characterized recently in LPS-treated RAW 264.7 cells by Wadleigh *et al.* [200] as discussed below.

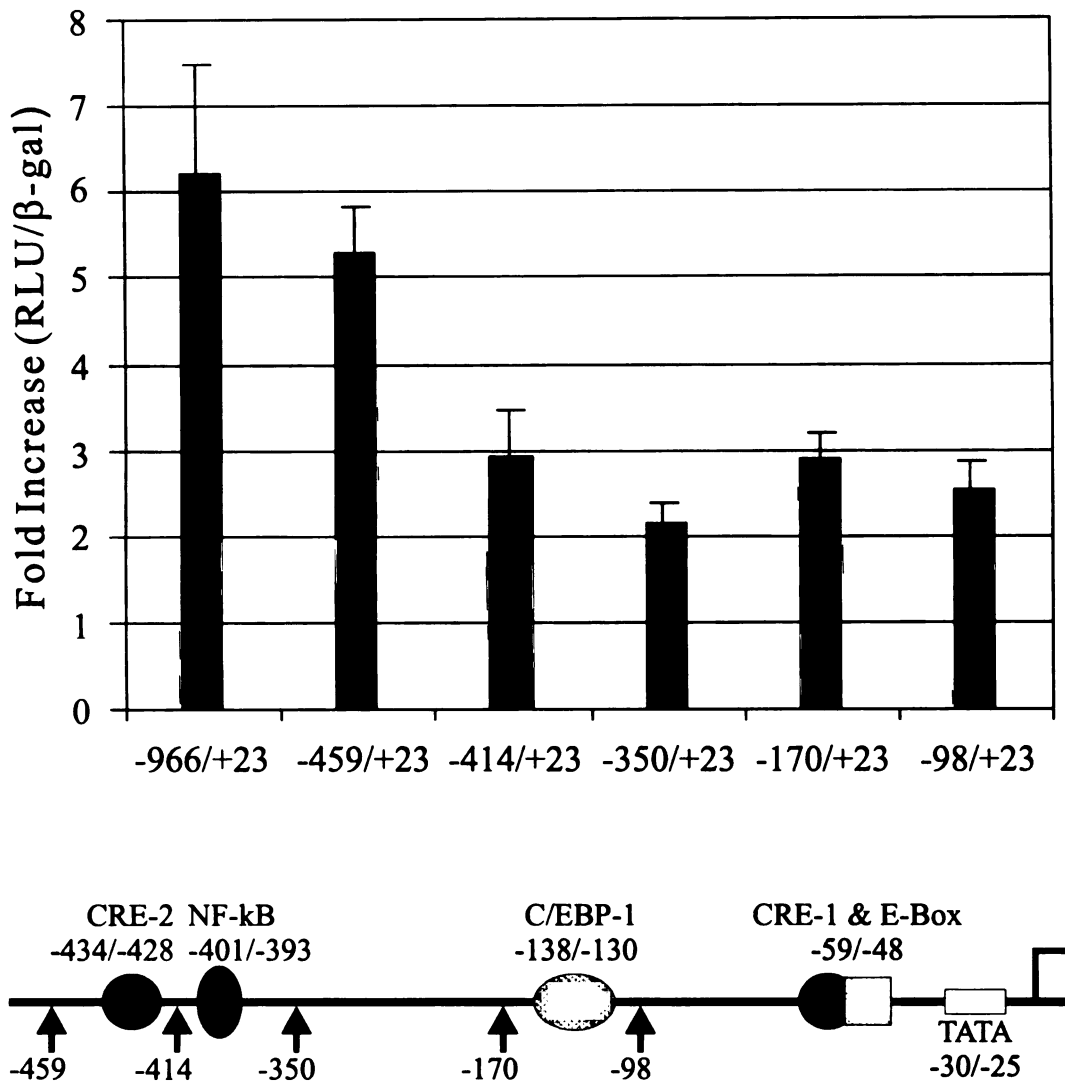


Figure 8. Promoter activity of COX-2 deletion constructs. Deletion of several different upstream response elements of the COX-2 promoter block efficient LPS-induced promoter responses. A nested set of deletions were prepared from the native 966/+23 COX-2 promoter. RAW 264.7 cells were transiently co-transfected with 2.5 μg luciferase promoter reporter plasmids containing different regions (-966 to +23) of the COX-2 promoter and 0.5 μg of a CMV β-galactosidase plasmid. Twenty-four hr post transfection the cells were stimulated for 12 hr with LPS (200 ng/ml). Data represent the relative increase in promoter activity between cells transfected with the same plasmid, normalized to β-galactosidase activity, with and without LPS treatment, from three independent experiments replicated in triplicate. Error bars represent the average of one standard deviation from control and LPS-stimulated cells.

To investigate the role of the CRE-2 relative to the previously characterized *cis*-acting elements, we engineered and analyzed mutations in the *cis*-acting CRE-1 (-59/-52), C/EBP-1 (-138/-130), NF-κB (-401/-393), and CRE-2 (-434/-428) in the COX-2 promoter (–966 to +23) fused to a luciferase reporter gene (Table III). The results are summarized in Figure 9. The data represent the relative light intensity from luciferase activity normalized to β-galactosidase activity expressed by a separate pCMV-βgal plasmid. Mutations in any one of these four response elements resulted in significant ($p<0.05$) decreases in the levels of inducible promoter activity. The decrease in promoter activity caused by the mutation of CRE-2 was similar in magnitude to those caused by individual mutations in the NF-κB element or the CRE-1. Curiously, mutations of both the NF-κB and CRE-2 caused a decrease in promoter activity that was not significantly different from the decrease caused by single mutations of either of these sites ($p < 0.05$). This latter result was surprising because the mutation of CRE-2 in combination with CRE-1 had synergistic effects.

The response element mutation constructs were used in time course experiments along with the wild type –966/+23 promoter construct and a shorter –98/+23 promoter reporter plasmid. In these experiments the six plasmids were transfected to individual wells of a 6 well plate. LPS was added at the time points indicated in Figure 10. The data are presented as relative light units from luciferase assays of cellular lysates for each time point. These data indicate that over the period of the time course, the mutations have similar effects relative to the wild type promoter control.

CRE-2 is bound specifically by nuclear proteins. Electrophoretic mobility shift assays established that a ^{32}P-labeled 26 bp double-stranded DNA probe containing the

Table III. Oligonucleotides used for EMSAs and mutants

Sequence of the primers used for EMSA probes and the generation of mutant promoter reporter constructs. Mutated bases are in lower case. 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'
C/EBP-1	5'-CGCTGCGGTTCTTGCGCAACTCACTG-3'
Mutant C/EBP-1	5'-CGCTGCGGTTCccGCtCAACTCACTG-3'
CRE-1	5'-GAGTCACCACTACGTCACGTGGAGTC-3'
Mutant CRE-1	5'-GAGTCACCACTAatTCACGTGGAGTC-3'

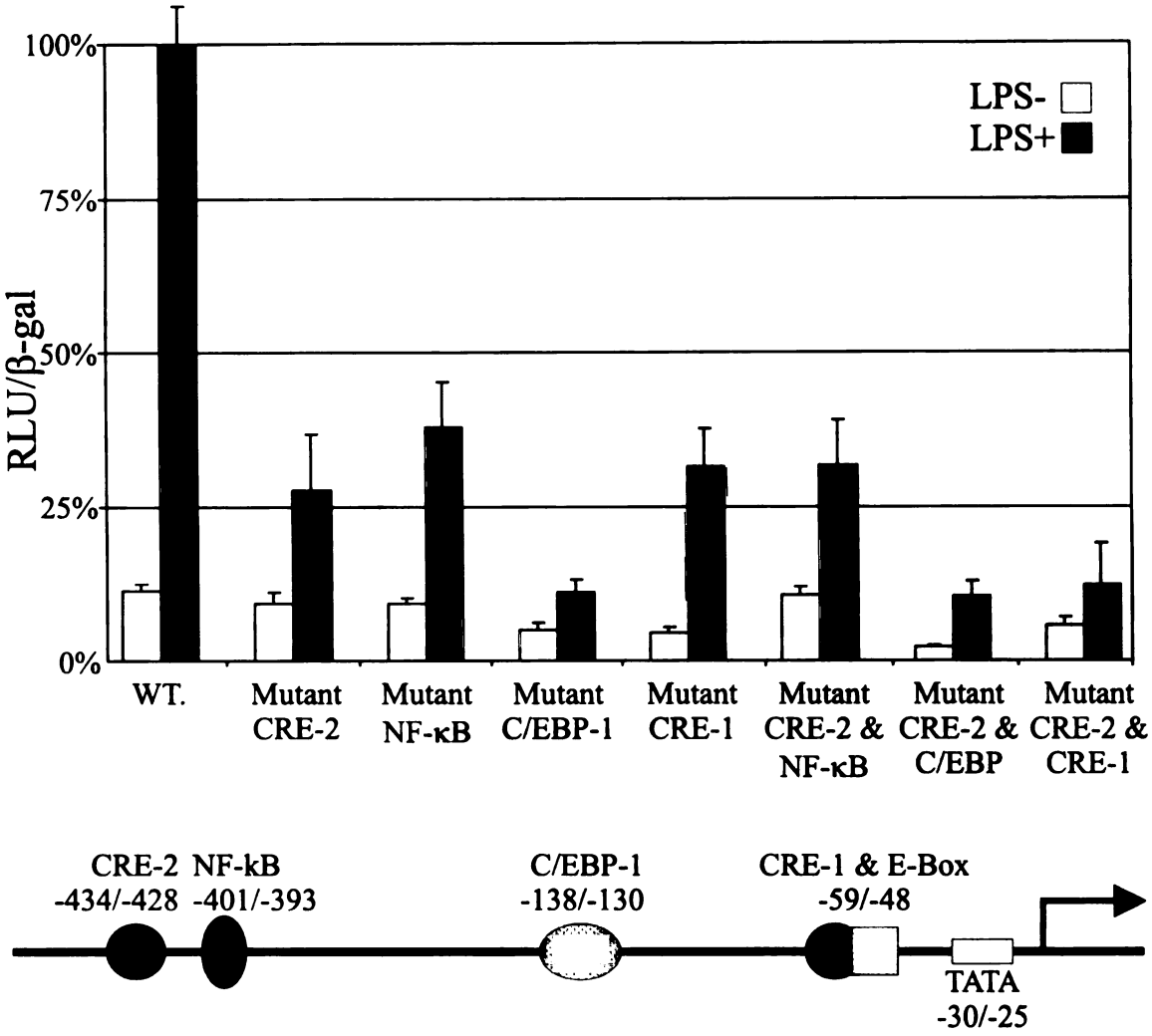


Figure 9. Promoter activity of COX-2 promoter mutation constructs. Mutations of several different response elements of the COX-2 promoter block efficient LPS-induced promoter responses. RAW 264.7 cells were transiently co-transfected with 2.5 μg luciferase promoter reporter plasmids containing different regions (-966 to +23) of the COX-2 promoter and 0.5 μg of a CMV β-galactosidase plasmid. Twenty-four hr post transfection the cells were stimulated for 12 hr with LPS (200 ng/ml). Data represent normalized means of three independent experiments performed in triplicate. The solid bars represent LPS-stimulated and unstimulated treatments, respectively. The error bars represent one standard deviation of the mean. Luciferase activity was normalized to units of β- galactosidase activity.

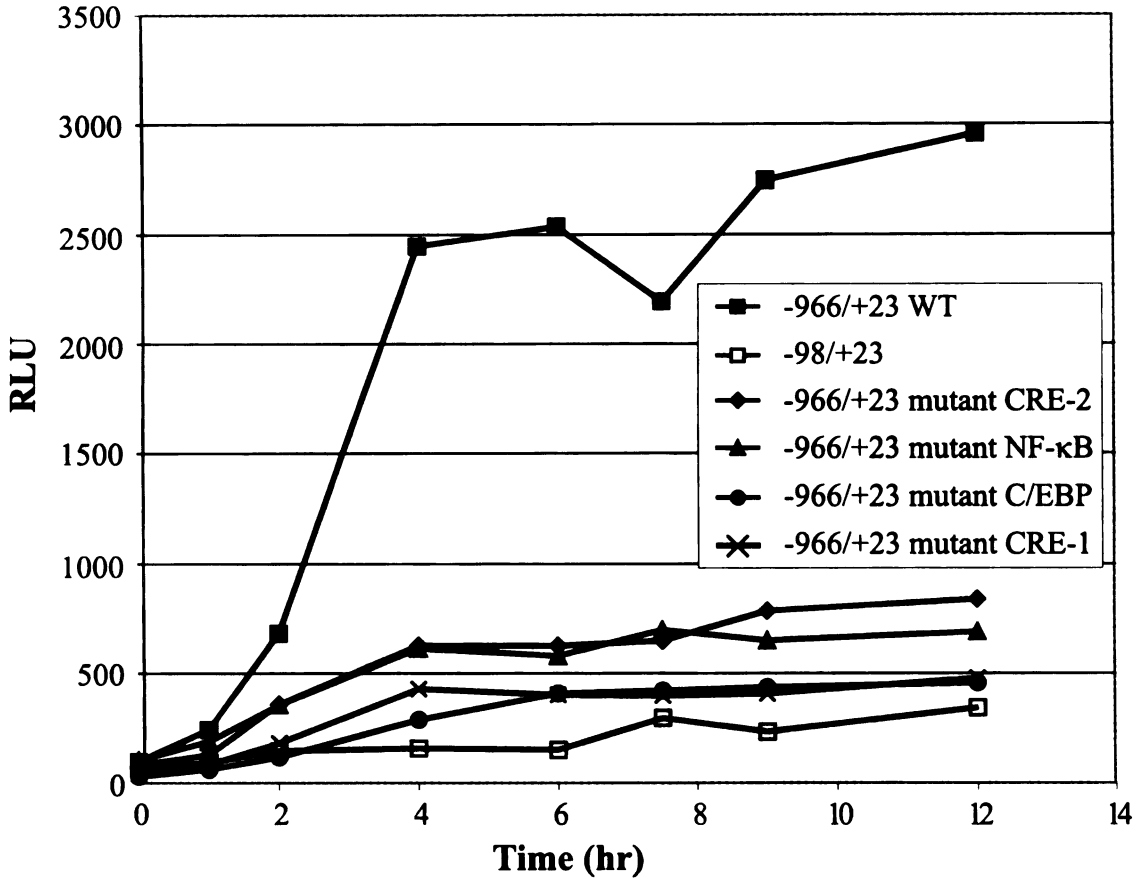


Figure 10. Time Course with the mutant promoter reporter plasmids. Mutations in the CRE-2, NF-kB, C/CBP and CRE-1 response elements block promoter activity. This effect is observed to a similar extent over the time course of this experiment. RAW 264.7 cells were transfected with 2.5 μg luciferase promoter reporter plasmid. Twenty-four hr post transfection, the cells were stimulated with LPS (200ng/ml) for 12 hours.The data represent the luciferase activity measured in cell lysates from each time pont, measured in relative light units (RLU).

CRE-2 was bound by proteins from nuclear extracts of both control and RAW 264.7 cells that were stimulated with LPS for either 1 or 12 hr. This binding was eliminated by competition with a 25 fold molar excess of unlabeled DNA probe but was not eliminated by a non-specific (NF-κB consensus) probe or a mutant CRE-2 probe (Figure 11; Table III). The binding of nuclear proteins to the CRE-2 is not inducible or downregulated by LPS. That is, the intensity of the radioactive CRE-2 band was similar in nuclear extracts from control and LPS-stimulated cells, indicating that the CRE-2 response element is bound constitutively.

Supershift assays with an anti-CREB antibody that can interact with CREB, ATF-1 or CREM transcription factors, indicated that the CRE-2 is bound constitutively by a member of the CREB/ATF transcription factor family (Figure 12). In supershift assays with an antibody that recognizes phospho-CREB, a small amount of the CRE-2 probe was shifted with equal intensities at each of the three time points. This result indicates that a fraction of the CREB is phosphorylated and provides additional evidence for the identity of the CRE-2 binding factor as CREB. We also used an antibody specific for the CBP and found that this antibody caused the shift of a low mobility complex. When antibodies to c-Fos or AP-1 (Jun) were used, no supershift or inhibition of binding to the CRE-2 probe were observed (data not shown).

NF-κB binding complex. Because the NF-κB response element is necessary for maximal promoter activity, we characterized the binding of nuclear proteins to this response element. A DNA probe for the NF-κB *cis*-acting element was bound in a lower mobility complex by nuclear proteins from cells treated for one hr with LPS and in a more mobile complex by nuclear proteins from cells treated for 12 hr with LPS

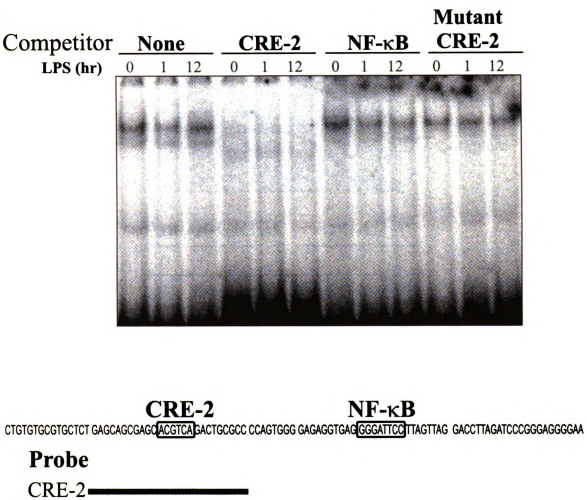


Figure 11. Specific binding of nuclear protein to the CRE-2 probe. Nuclear extracts were prepared from unstimulated RAW 264.7 cells or cells stimulated with LPS (200 ng/ml) for 1 or 12 hr. Double stranded ^{32}P-labeled CRE-2 probe was incubated with each of the three extracts in the presence and absence of 25 molar excesses of unlabeled CRE-2, NF-κB (non-specific competitor), or mutant CRE-2 double stranded probes as described in the text.

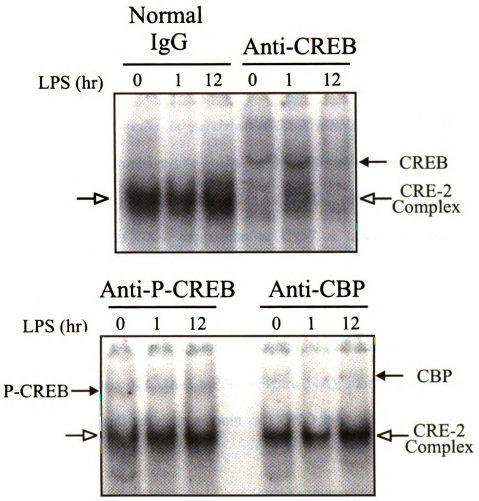


Figure 12. CREB and CBP bind the CRE-2 Probe. The CRE-2 probe is bound by anti-CREB-1, anti-phospho-CREB, and anti-CBP. EMSA supershift assays were performed with a ^{32}P-labeled CRE-2 probe and nuclear extracts from RAW 264.7 cells stimulated with LPS (200 ng/ml) for 0, 1 or 12 hr in the presence of a normal IgG control antibody or antibodies to CREB-1, phospho-CREB, and CBP as described in the text. The solid arrows beside the EMSA autoradiograms indicate the supershifted complexes. The open arrows indicate the complex that binds the CRE-2 probe.

(Figure 13). Supershift assays were performed with antibodies to p50 or p65 Rel/NF-κB proteins involved in the different dimeric NF-κB complexes. The anti-p50 antibody caused shifts in both the high and low mobility complexes, but the anti-p65 antibody shifted only the low mobility complex (Figure 13). These data indicate that the NF-κB response element of the COX-2 promoter is bound predominantly by a p50/p65 heterodimeric complex in the early phase of cell activation by LPS and predominantly by a p50 homodimer in the late phase.

LPS treatment results in persistent COX-2 mRNA synthesis. Stimulation of macrophage and macrophage-like cells with LPS results in the synthesis of prostaglandins and the prolonged expression of COX-2 protein [195, 196, 210, 211]. Because LPS induced COX-2 expression in U937 is heavily influenced by post-transcriptional regulation[212] , and because p50 dimers are associated with negative regulation of inflammation related genes [213], we performed nuclear run-on assays to assess the level of COX-2 transcription. We found that LPS stimulation resulted in rapid and prolonged synthesis of COX-2 mRNA (Figure 14A); moreover, the rate of mRNA synthesis correlated well with the amount of COX-2 mRNA observed in RAW 264.7 cells (Figure 14B).

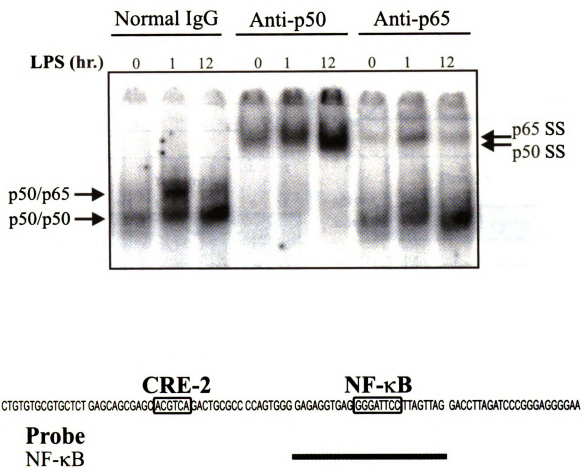


Figure 13. p65 and p50 bind the NF-κB probe. Nuclear extracts were prepared from control or LPS-stimulated RAW 264.7. The cells were stimulated with LPS (200 ng/ml) for 0, 1, or 12 hr. Double stranded, ^{32}P-labeled NF-κB probe was incubated with nuclear extract and either normal IgG control serum, anti-p50 or anti-p65 antibodies as described in the text.The NF-κB probe complexes with p50/p65 and p50/p50 transcripton factors are indicated with arrows on the left side of the figure. Supershifted (SS) complexes bound with antibody to p65 or p50 are indicated with arrows on the right side of the figure.

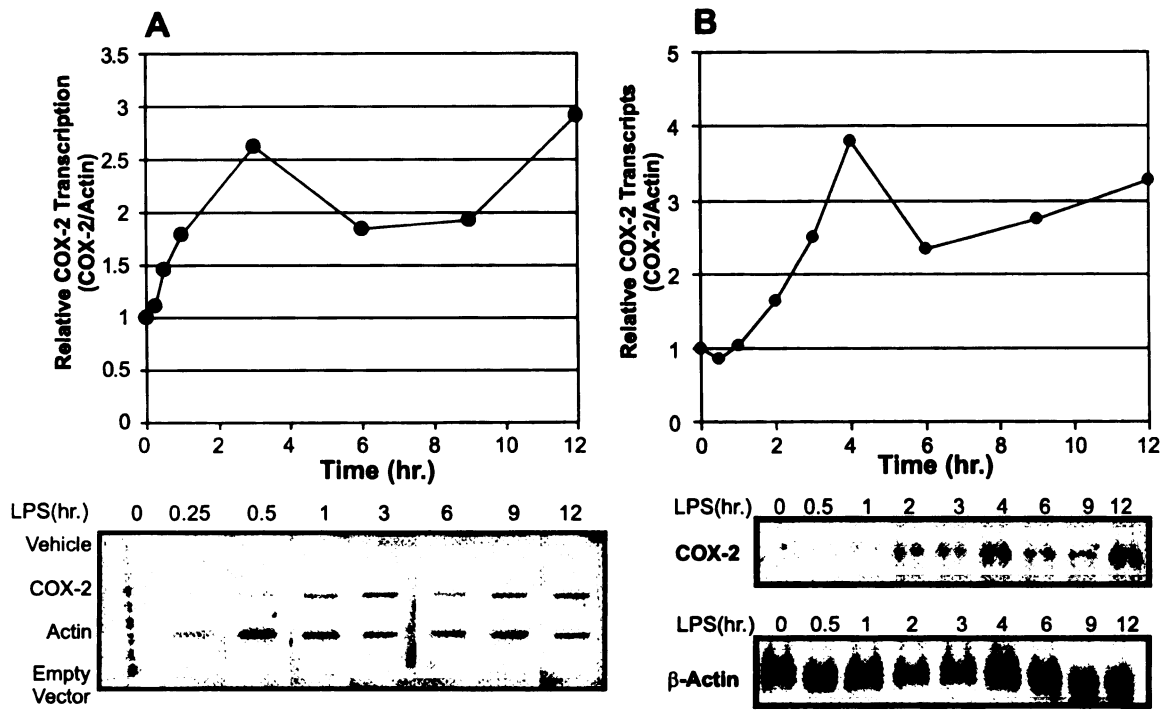


Figure 14. Nuclear run-on and Northern blot analysis of LPS-stimulated RAW 264.7 cells. **A)** Nuclei were isolated from cells stimulated with LPS (200 ng/ml) for the indicated times. The nuclei were incubated with [^{32}P]UTP to label newly synthesized transcripts as described in the text. RNA was isolated and blotted onto a nitrocellulose membrane and with vehicle, mCOX-2 cDNA, -actin cDNA or an empty vector DNA control. The membrane was washed and exposed to a phosphoimaging screen. Densitometry was performed with Image Quant software. B) Northern blot analysis was performed as detailed in the text. RNA was isolated from 100 mm plates of RAW264.7 cells stimulated with LPS (200ng/ml) for the indicated times. Total RNA (15μg) was separated on a 0.8% agarose, 4% formaldehyde TAE gel, transferred to a nitrocellulose membrane, and hybridized to mCOX-2 and β-Actin cDNA probes.

Discussion

The induction of COX-2 gene transcription observed in fibroblasts in response to mitogens, serum, and transformation requires only the first 80 bp of the COX-2 promoter [64, 65]. A larger portion of the COX-2 promoter is necessary for maximal COX-2 gene expression in macrophages [155, 200, 214], osteoblasts [102, 109], and endothelial cells [92].

The current model for the transcriptional regulation of the COX-2 gene in LPS-stimulated macrophages includes LPS activation of the toll-like receptor (TLR4), which, in turn, initiates signaling through MyD88/IRAK/TRAF6/ECSIT resulting in the activation of ERK, JNK, p38, PKC, and NIK. These kinases exert their actions by phosphorylating either transcription factors or downstream effectors to cause the transcriptional machinery to begin transcribing the COX-2 gene.

The *cis*-acting elements of the murine promoter that are necessary for a response to LPS include an overlapping CRE (CRE-1) and E-box (-59/-48) [215] and two C/EBP sites at –138/-130 (C/EBP-1) and –93/-85 (C/EBP-2) [200] (Figure 7). AP-1, CREB or USF-1 transcription factors bind to the overlapping CRE-1 and E-box, and C/EBP transcription factors bind to the C/EBP response elements [92]. We have now established that both the NF-κB response element at -401/-393 and a second CRE (CRE-2) located at -447/-440 are also necessary for maximal LPS induction of the murine COX-2 gene. We have also shown that p65 and p50 bind the NF-κB site in different combinations and that CREB and CBP can associate with the CRE-2 site. This collection of *trans*-activating factors may function independently or cooperatively as an enhancer complex with the general transcriptional machinery to activate the COX-2 gene (Figure 15).

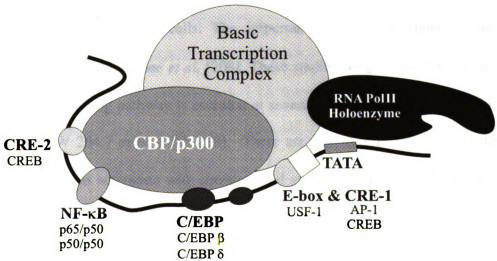


Figure 15. Model for the interactions of *trans*-activating factors associated with the COX-2 promoter.

NF-κB response element in LPS-induced COX-2 gene expression. There are several lines of evidence that suggest that the NF-κB response element and NF-κB signaling play a role in activating the COX-2 gene. The NF-κB response element is conserved and is in a similar location in the human, monkey, equine, bovine, rat, and mouse promoters. The consensus sequence is nearly identical and follows a (5'-GGGATYCCC-3') motif that has been found to favor interactions with p50 homodimers [213]. The κB1 site of the IP-10 gene even exhibits induced NF-κB binding with a time dependent change in composition [216] (i.e. the composition of the binding complex changes from a p65/p50 heterodimer to a p50 homodimer at 3 hr post LPS-treatment) in LPS stimulated RAW 264.7 cells. The importance of NF-κB signaling has been demonstrated recently by Rhee *et al.* [208]. These studies suggest that NF-κB activation via the TLR4 signaling pathway is essential at several steps for induced COX-2 reporter activity in RAW 264.7 cells. [207, 208]. There are also several reports indicating that inhibition of NF-κB activation with chemical and synthetic peptide inhibitors and decoy oligonucleotides block COX-2 activation [202, 204, 205, 217, 218].

Recently, Wadleigh *et al.* [200] reported that there was no requirement for the NF-κB site or for NF-κB activation for the LPS stimulated activation of a murine COX-2 promoter reporter in RAW 264.7 macrophages treated with LPS. Our present results indicate that when the NF-κB response element is mutated, over half of the COX-2 promoter activity is lost. Binding to the NF-κB response element is inducible, and the composition of the factors binding at this site changes from a p65/p50 complex to a p50 homodimer at 1 and 12 hr after LPS stimulation.

The differences in the results of the promoter activity assays observed here and by Wadleigh *et al.* could be due to the time course of the experiments, the amount of LPS used, the design of the promoter reporter construct or perhaps subtle differences in the RAW 264.7 cells. Wadleigh *et al.* stimulated their RAW 264.7 cells with LPS for 5 hours with 10 ng/ml LPS using a plasmid containing –724/+7 of the murine COX-2 promoter. We stimulated RAW 264.7 cells with LPS for 12 hours with 200 ng/ml LPS using a plasmid containing –966/+23 of the murine COX-2 promoter.

To check for a time dependent requirement of the CRE-1, C/EBP, NF-κB, and CRE-2 response elements, we performed a time course experiment with the wild type –966/+23 promoter construct with mutations in these four response elements and with a construct containing only –98/+23. (Figure 10) We found that each mutation had a similar effect at time points between 1 and 12 hours. Each response element mutation significantly decreased the level of induced promoter activity across the time course.

RAW 264.7 cells are exquisitely sensitive to LPS, and as a consequence there is only a small increase in inflammatory cytokine release [219] caused by an increase from 10 ng/ml to 100 ng/ml of LPS. It is likely that the differences in the LPS doses used in our experiments only resulted in a small increase in promoter activity over what was observed by Wadleigh *et al.*

The promoter constructs used in our experiments and by Wadleigh *et al.* differ by 242 bases on the 5' end. It is unlikely that this difference resulted in the large difference between our results, because in Figure 8 the promoter construct containing –966/+23 and –459/+23 had similar levels of induced promoter activity. The 16 bp difference at the 3'

end of the construct is also unlikely to have a large affect on promoter activity, because this is a relatively short region that does not contain any identified response elements.

In the experiments by Wadleigh *et al.*, LPS treatments result in about a 4 fold increase in promoter activity. In our experiments, we routinely observe 6 to 12 fold increases in promoter activity. We have observed that after culturing RAW 264.7 cells for 4 to 6 months (about 50 to 60 passages), LPS induced COX-2 promoter activity drops to between 2 and 4 fold. We prepared a working cell bank of RAW 264.7 cells from a fresh ampule of cells obtained from the American Tissue Type Collection. We reinitiated our RAW 264.7 cell cultures at about 5 month intervals or when induced promoter activity decreased below about 5 fold.

EMSA experiments are used to approximate what transcription factors may bind to a response element. The actual condition within cells on the promoter in the context of chromatin may differ significantly. EMSAs show what can be reconstituted on a short piece of naked DNA from proteins extracted from the nucleus of a cell. In our experiments we observed that a p50 dimer associates with the NF-κB response element during the late phase of the LPS response. If this condition also exists on the native promoter it could cause the down regulation of transcription by displacing the transcriptionally active p65 containing complex. Alternatively the p50 dimer may play some role in the persistent expression of COX-2 or have only a neutral effect.

The same signaling pathways that activate the transcription of COX-2 in LPS-treated RAW 264.7 cells are also involved in the activation of the TNF-α gene [142, 220, 221]. LPS-induced COX-2 activity, expression, and mRNA levels are upregulated for 24 to 48 hr. Similar results are observed with the TNF-α gene, although the time course is

shorter. The rate of TNF-α gene transcription is decreased to background levels within hours of the initial activation, but because the mRNA is stabilized by way of a p38 mediated mechanism, mRNA and protein expression levels remain elevated for at least 12 to 18 hr after LPS treatment [222, 223]. The downregulation of TNF-α gene expression after LPS stimulation results in part from increased expression of p50 and a resultant increase in the binding of p50 dimers to NF-κB response elements in the TNF-α gene promoter [213]. Dimers of p50 commonly function as repressors of transcription in other promoters as well [158, 159].

NF-κB dependent gene expression involves transcriptional coactivators that are proposed to function by bridging sequence specific transcription factors to the basal transcriptional machinery [224]. p65 and c-Rel are the transcriptionally active members of the Rel family of transcription factors [159]. Activated p65 associates with CBP to *trans*-activate NF-κB dependent genes [225]. The p50 and p52 Rel transcription factor members are transcriptionally inactive as dimers and have been thought to act as transcriptional repressors [158, 159]. This paradigm, however, has recently been challenged by the observation that the p160 family of transcriptional co-activators are also involved in NF-κB dependent gene activation. The steroid co-activator-1 (SRC-1) has been found to interact with p50 to potentiate p65 independent NF-κB-mediated transactivation [181]. Because p50 is not involved in downregulating COX-2 transcription, it may be involved in the persistent transcription of COX-2; however, in our hands, overexpression of p50 did not have a significant effect on LPS induced promoter activity, a result also observed by Yamamoto *et al.* [102] with TNF-α stimulated MC3T3-E1 cells.

Monocyte and macrophage cells have been shown to produce prostaglandins and express COX-2 for 24 to 48 hr [195, 196, 210, 211] in response to pro-inflammatory stimuli. Persistent expression is most likely due to prolonged transcriptional activation because of the instability of the COX-2 mRNA. Recently, however, several studies in monocytes and macrophages have revealed that post-transcriptional regulatory mechanisms result in mRNA stabilization that can lead to sustained COX-2 expression [212, 226].

Because of the possibility that post-transcriptional stabilization of COX-2 mRNA may account for the prolonged expression of COX-2, and because a known transcriptional repressor, the p50 homodimer, binds the NF-κB response element after the initial activation of the COX-2 gene, we performed nuclear run-on experiments to determine whether transcription of the COX-2 gene was downregulated after LPS treatment. We found that the initial rate of transcription was higher than the steady state rate of transcription observed after several hours of LPS stimulation. This pattern of gene transcription correlated well with the pattern of mRNA accumulation. These data establish that the COX-2 gene is persistently activated in response to LPS, although post-transcriptional regulatory mechanisms may also contribute to the overall response.

In promoter activity assays, we observed a higher level of inducibility than in the nuclear run-on and Northern blot experiments. The level of actin transcription was increased by the LPS treatments, which lowers the relative increase in COX-2 levels that are reported in Figure 14. The relatively stable luciferase protein and mRNA enhance the measured levels of increased activity, and probably most importantly, the transfected

promoter reporter plasmid is not integrated into the chromatin like the endogenous promoter.

CRE-2 in LPS-induced COX-2 gene expression. Mutations made in the CRE-2 site caused a decrease in promoter activity that was similar in magnitude to the effects caused by mutation of the NF-κB and CRE-1 response elements but was not as potent as the effect caused by the C/EBP site mutation. Double mutations at CRE-2 and the other three response elements revealed additive decreases in promoter reporter activity when the CRE-2 mutation was combined with the C/EBP and CRE-1 mutants. Interestingly, however, the double CRE-2 and NF-κB mutation did not cause an additive decrease in promoter activity. Since both the CRE-2 and NF-κB mutation mutants resulted in similar decreases in promoter activity, it is possible that each one mediates a similar level of transcriptional activation. However, when both sites are mutated, the mutations do not cause an additive decrease in promoter activity. Because there is no additive effect, the sites most likely do not act independently of each other. We suggest that the combination of the factors at the CRE-2 and NF-κB sites are required to mediate their *trans*-activation potential. This type of interaction is cooperative, not in the sense of binding DNA, but in causing an increase in transcriptional activation when both sites are intact.

Binding of nuclear proteins to the CRE-2 is constitutive, but because removal or deletion of the CRE-2 decreases the promoter activity, we expected either binding or phosphorylation of CREB at this site would be inducible. The CREB transcription factors generally bind DNA independently of activation and are phosphorylated to an active state. In supershift experiments, the nuclear protein complex bound to the CRE-2 probe was constitutively shifted by antibody that is specific for the conserved carboxyl terminal

domain of CREB and ATF transcription factors. Antibody against phosphorylated CREB also produced a constitutively shifted band, further confirming the presence of CREB bound to CRE-2. Because CREB is bound to CRE-2, we also used antibody against CBP to test for the presence of this factor and found that a lower mobility/probe binding complex was supershifted. The intensity of the shifted bands observed with the anti-phospho-CREB antibody is significantly less intense that what is observed with the anti-CREB antibody. It is likely that only a small portion of the CREB in the nuclear extracts is phosphorylated, and this would cause a reduced signal. The supershifted CBP complex is also much less intense that the anti-CREB supershift. Any signal observed in this experiment is dependent on the extraction of this large protein from the nucleus, and the assembly of a DNA probe-DNA binding protein-CBP complex. These two requirements decrease the probability of observing this interaction.

CBP has been suggested to play a role in the transcriptional regulation of COX-2 but has not previously been shown to interact with any of the COX-2 promoter [64]. CBP and other transcription co-activators are found in limited amounts within cells and are competed for by ligand bound nuclear hormone receptors and other inducible transcription factors [85, 86]. This model was recently examined in the context of the COX-2 promoter with PMA-treated epithelial cells [90]. PPARγ ligands, ciglitazone, and 15-deoxy-$\Delta^{12,14}$prostaglandin J_2 inhibit the PMA response, which is almost completely rescued by co-transfection with c-Jun and CBP and partially rescued by CBP alone. This suggests that there is a functional interaction between CBP and *trans*-activating factors bound to the promoter. Together, the results of this study and our current findings

provide evidence that CBP interacts both functionally and physically with the COX-2 promoter.

Conclusion. We have identified a new *cis*-acting element (CRE-2) functional in the COX-2 promoter during LPS induction of COX-2 expression in the RAW 264.7 cell line. This CRE-2 element is bound specifically by a CREB/ATF transcription factor along with CBP. These complexes appear to bind the CRE-2 probe constitutively. We also observed that the NF-κB response element is inducibly bound primarily by p65/p50 after 1 hr LPS stimulation, and primarily by p50/p50 after 12 hr. Promoter reporter assays suggest the CRE-2 and NF-κB response elements act together to facilitate a maximal LPS induced response. Our data corroborate the model of Mestre *et al.* [155] that multiple redundant signaling pathways lead to the activation of COX-2 gene transcription and the observations of Rhee *et al.* [208] that NF-κB activation is required for maximal COX-2 expression.

CHAPTER 3

EXAMINATION OF THE COFACTORS ASSOCIATED WITH THE CRE-2 AND NF-κB REGION

Introduction

The purpose of this section is to document several follow-up experiments that were designed to answer questions raised by the data presented in the previous chapter. Based on our new data and what is known about the transcription factors that are involved in this system, we developed a model for the CRE-2 and NF-κB region of the COX-2 promoter (Figure 16). Before the addition of LPS, the CRE-2 site may be occupied by a CREB and CBP complex. Immediately after LPS stimulation, an NF-κB transcription factor complex comprised of p50 and p65 binds to the NF-κB site. In the later phase, the gene is still transcriptionally active and the NF-κB site is bound by a p50 homodimer.

The late phase of transcription is unique because there are many examples of transient activation of COX-2. Since this upstream region is not necessary for transient activation, we considered the possibility that it may play a role in observed prolonged transcription. Of particular interest was the p50 homodimer that is present during the late phase. Activation of NF-κB is generally transient, but there are exceptions, as was discussed in Chapter 1. It is possible that I-κBβ may be bound to the p50 dimer which prevents it from being removed from the promoter. While the p50 occupation may be neutral, we considered the possibility that p50 was involved in either repression or activation during the late phase of transcription. To test for repression we performed

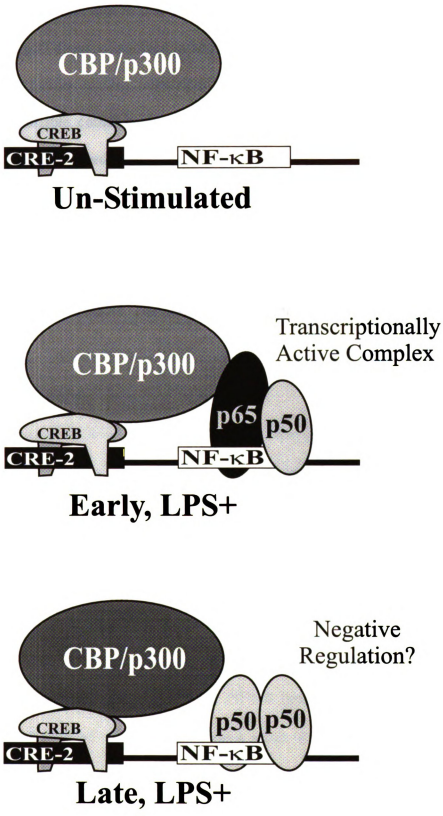


Figure 16. Model of the CRE-2 and NF-κB region during LPS activation

nuclear run-on assays, and as discussed in Chapter 2, transcription occurs for up to 12 hours. If the p50 dimer is involved in activation, it does not act alone. Since the NF-κB transcription factors require transcriptional coactivators to initiate transcription, we looked for possible cofactors that may be involved in the activation process. These experiments are described below.

Since our data indicated that the CRE-2 and NF-κB sites function together, we considered the possibility that a large complex may form on the combined CRE-2 and NF-κB sites. We thought that this complex might be visible as a very low mobility DNA binding complex in EMSAs. The design of this experiment and the results are described below.

Supershift with I-κBβ

The persistent activation of NF-κB is mediated in some cases by I-κBβ, as was discussed previously in chapter 1. In LPS-stimulated RAW 264.7 cells, I-κBβ is rapidly degraded and then re-synthesized [140]. Since COX-2 gene transcription is persistently activated by LPS and I-κBβ is present in RAW 264.7 cells, we performed EMSA supershift assays to determine if I-κBβ was part of the p50 homodimer-containing complex bound to the NF-κB response element during the late phase of the LPS response.

Results. Antibody against I-κBβ did not cause a supershift or blockade of the complex bound to the NF-κB probe in nuclear extracts from either the control or the LPS-stimulated RAW 264.7 cells (data not shown).

Discussion. This experiment was aimed at understanding how the p50 homodimer was maintained on the COX-2 promoter during the late phase of the LPS response. We could not detect I-κBβ as a component of the complex that binds the NF-κB probe. We used the supershift EMSA technique because it is reasonably sensitive to small amounts of protein and because we could compare the results of this assay with those of other similar experiments. Other methods could also be used to detect this interaction. For example, immunoprecipitation experiments could be useful for determining if I-κBβ associates with the NF-κB proteins that we have identified as interacting with the NF-κB response element probe in the nuclear extracts from LPS-stimulated RAW 264.7 cells. Chromatin immunoprecipitation with I-κBβ antibody could be used to detect interactions between I-κBβ and the COX-2 promoter. Since this latter

technique uses PCR amplification, it may be a more sensitive probe for this type of interaction.

Alternatively, another I-κB protein may be involved in this complex. Bcl-3 has been observed to bind p50 homodimers. The role of Bcl-3 is not well understood. In some cases it appears to be involved in facilitating NF-κB transcription by removing p50 dimers from promoters [180]. In other instances, it appears to function as a coactivator of NF-κB dependent transcription. Before looking for interactions between the NF-κB response element and Bcl-3, the presence of a murine Bcl-3 homologue in RAW 264.7 cells needs to be verified.

Co-Transfection Experiments with Transcription Factors and Transcriptional Coactivators

We sought to determine the functional significance of an apparent cooperative effect between the CRE-2 and NF-κB sites, the association of CBP and CREB with CRE-2, and the inducible binding of p65/p50 and persistent occupancy of the NF-κB site by a p50 homodimer. One possibility is that transcriptional activation by NF-κB requires multiple coactivators. [224] CBP is a transcriptional coactivator of p65 [225], and a CBP and SRC-1 are transcriptional coactivators of p50 [181]. Because of the arrangement of CRE-2 and the NF-κB sites on the murine COX-2 gene, our other data suggest that LPS stimulation brings CBP and p65 into close proximity. The implications of this relationship are similar to those explored by Gerritsen *et al.* in their experiments with the E-selectin promoter [225]. The E-selectin promoter contains three regulatory domains. The first contains an element that is recognized by CREB/ATF and AP-1 transcription factors and the second two elements are bound by p50/p65 NF-κB heterodimers. (Both the E-selectin and COX-2 promoters have a similar CRE and NF-κB pair in a region approximately 500 bp upstream of the transcriptional start site.) The experiments by Gerritsen *et al.* [225] demonstrate that CBP and p300 physically interact and that this interaction is required for *trans*-activation of an E-selectin promoter reporter.

Results. To determine if CREB, CBP or SRC-1 could potentiate LPS-induced COX-2 activity, we obtained a CREB expression plasmid from Marc Montiminy (Salk Institute for Biological Studies, La Jolla, CA), a CBP expression plasmid from Richard Goodman (Vollum Institute, Oregon Health and Science Institute, Portland OR.) and pCR3.1-hSRC-1A expression plasmid from Bert O'Malley and Ming Tsai (Baylor

College of Medicine, Houston TX). Using co-transfection experiments, we determined if any of these factors alone or in combination could augment LPS-induced promoter activity. The transfection experiment was performed twice, in duplicate, with similar results. Figure 17 shows the results from one of the replicates. None of the factors, alone or in combination caused a dramatic increase or decrease in basal or LPS-induced promoter activity.

Previously overexpression of CREB has been shown to blocked LPS induced COX-2 promoter activity; however, we did not observe this negative regulation.

To determine if NF-κB transcription factors could potentiate LPS induced COX-2 promoter activity, we co-transfected p50 and p65 (obtained from Richard Schwartz, Michigan State University, East Lansing, MI.) alone and with the SRC-1a expression plasmid (Figure 18). The transfection was performed twice, in duplicate, with similar results. The results reported are from one representative set of transfections. None of the experiments resulted in an increase of either the basal or induced level of promoter activity to a degree that suggests a substantial interaction.

Discussion. Co-transfection of the transcription factors and transcriptional coactivators that we expected to be associated with the upstream promoter region had small effects on the basal and LPS induced levels of COX-2 promoter activity. This may indicate that sufficient levels of these factors are already present in the cell such that addition of more factor has no effect. Alternatively these factors may not be important for the activation of COX-2 gene transcription.

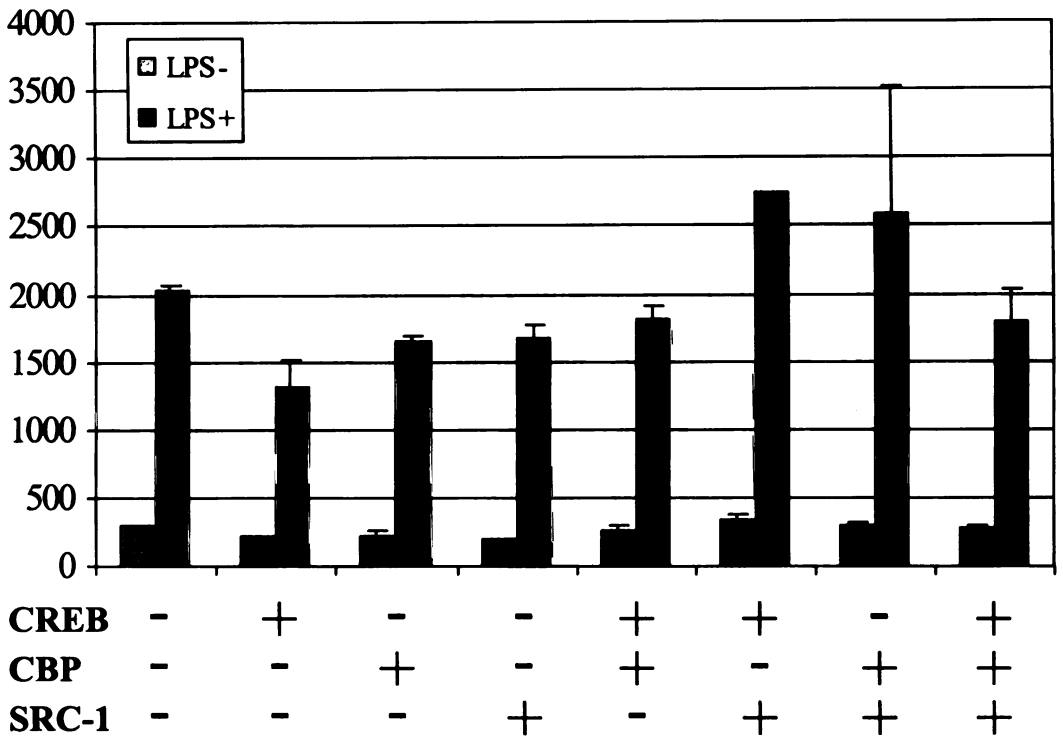


Figure 17. Co-transfection of the -966/+23 COX-2 promoter reporter with CREB, CBP and SRC-1. RAW cells were stimulated with LPS for 12 hr. The results are representative of two separate experiments prepared in duplicate.

Subbaramaiah *et al.* [90] observed that glucocorticoids block induced COX-2 promoter activity and that co-transfection of CBP and c-Jun restored promoter activity.Based on these observations, it may be necessary to disrupt the coactivator complex in order to detect an effect of adding the components back to the system. Methods of interrupting transactivation of the coactivator complex include pretreatment with glucocorticoids or PPARγ ligands or the addition of E1a adneovirus oncoprotein[6] [87, 89, 224, 227-229].

Another possibility is that the interactions between the factors bound at the CRE-2 and NF-κB sites are concentration dependent, where the binding of one factor is dependent on the binding of the other. This could be tested by overexpressing one factor and titrating the level of the other. If, for example, p65 binding was dependent on CREB occupation of the CRE-2 site, then increasing levels of CREB my enhance promoter activity in the presence of overexpressed p65.

When these experiments were performed I focused on the role of p50. It may be useful to further explore the role of p65 as an enhancer. If p65 and CBP form a transcriptionally active complex on the COX-2 promoter, it may be possible to enhance promoter activity by overexpressing combinations of p65, CBP, and SRC-1. Sheppard *et al.* found this combination to enhance E-selectin promoter activity [224].

[6] We have obtained an E1a expression plasmid (Nicholas Dyson and Fred Dick, Massachusetts General Hospital, Charlestown MA.) but have not yet completed these experiments.

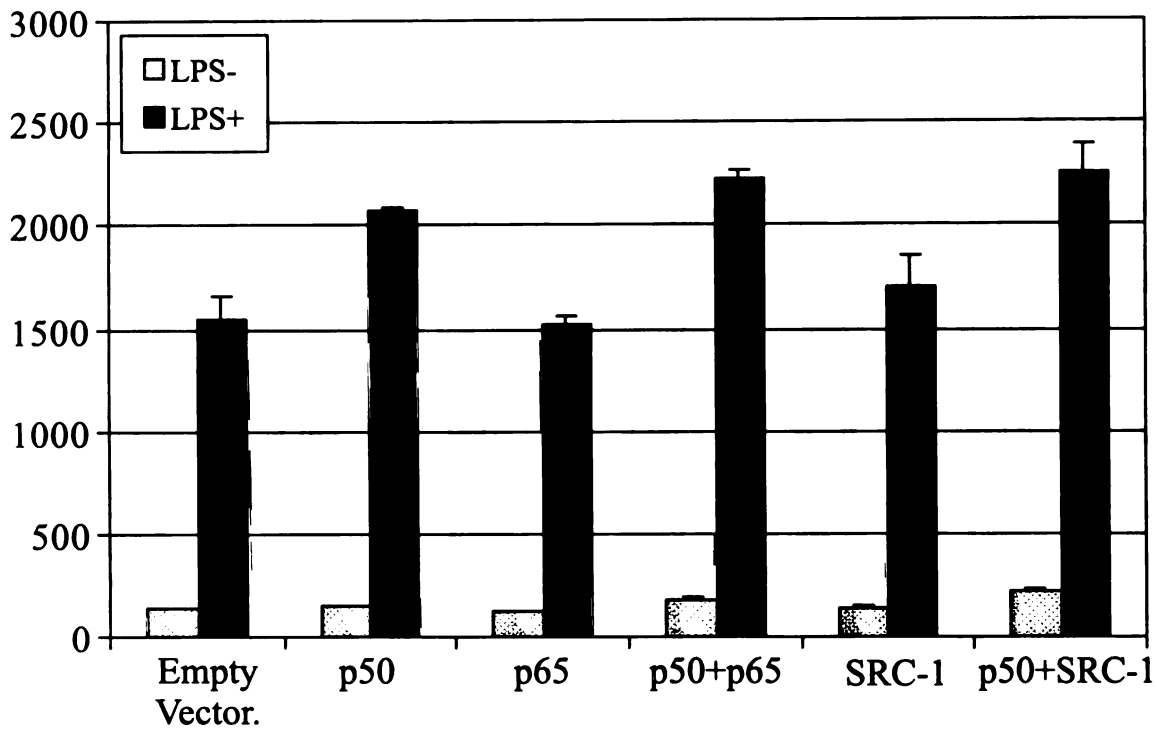


Figure 18. Co-transfection of the -966/+23 COX-2 promoter reporter with the plasmids for the expression of the NF-κB proteins p50 and p65 and the p160 family co-activator SRC-1. RAW cells were stimulated with LPS for 12 hr. The results are representative of two separate experiments prepared in duplicate...

Shifting for Cooperative Complexes

Promoter activity assays provided data leading to the assertion that the NF-κB and CRE-2 response elements mediate a cooperative effect on transcriptional activation. We thought that if the CRE-2 and NF-κB sites were on the same EMSA probe, we might be able to detect a very low mobility complexe that could be eliminated by the mutation of either the CRE-2 or NF-κB site.

Results. We constructed a 59 bp probe (–444 to –385) that contained both the CRE-2 and NF-κB sites, as well as constructs with a mutation in one or both sites. As a control, we also used a shorter probe based on the sequence between the CRE-2 and NF-κB sites (middle probe) and the two independent response element probes. The purpose of the middle probe was to verify that sequences between the CRE-2 and NF-κB response elements did not exhibit any additional unique shifted species (Figure 19).

We found that the 59 bp probe is bound by a complex that is dependent on the CRE-2 site that is not formed by the CRE-2 probe alone. This complex is most prominent at the 1 hr time point and is not visible at the 12 hr time point. (Figure 20).

Discussion. In all the previous EMSA experiments, we did not observe inducible binding or modification of the complex formed with the CRE-2 probe. In this experiment, we observed an inducible effect that is dependent on the CRE-2 site. This observation is not consistent with the model of a cooperative effect because the NF-κB mutation does not affect the low mobility band. It is difficult to explain exactly what this complex represents; however, the formation this type of low mobility complex indicates that a more highly ordered complex forms on this probe.

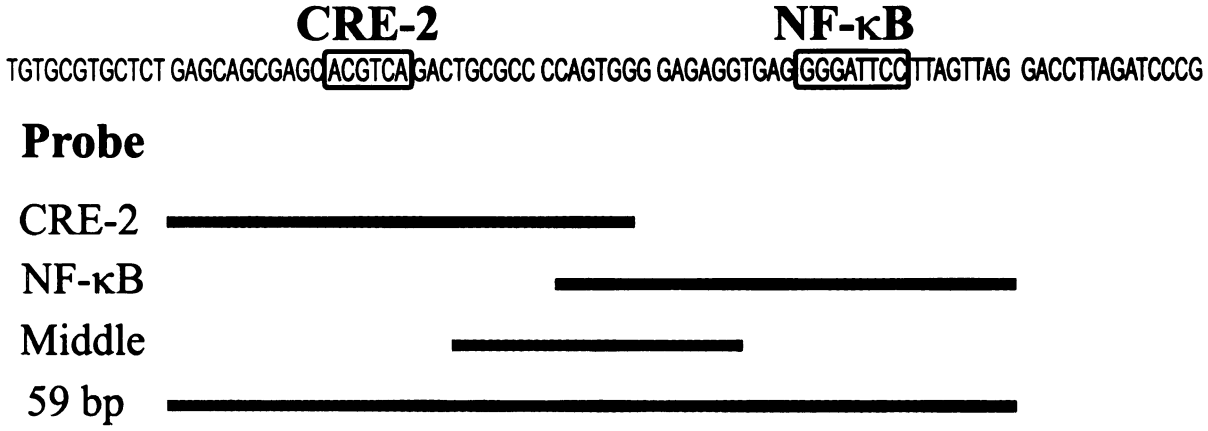


Figure 19. EMSA probe map

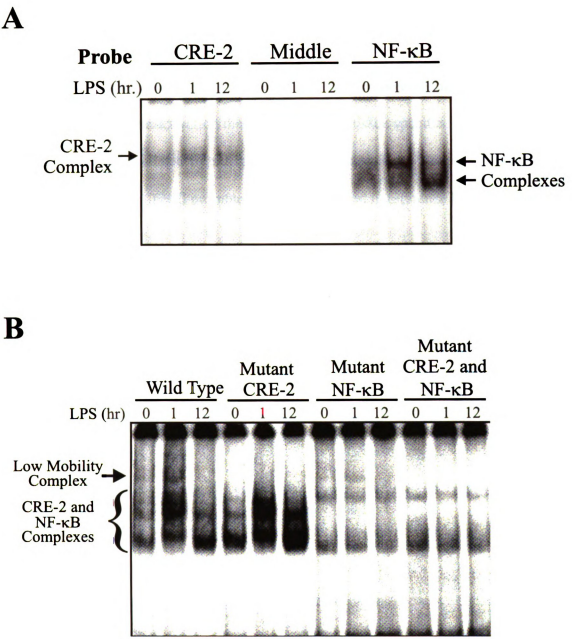


Figure 20. Comparison of shifted products between the site specific probes and a probe containing both the CRE-2 and NF-κB sites. A) EMSAs with the CRE-2, Middle, and NF-κBprobes. B) EMSAs with the 59 bp probe, containing the CRE-2 and NF-κB sites. Nuclear extracts were prepared from RAW 264.7 cell stimulated with LPS (200 ng/ml) for 1 or 12 hours. Binding with ^{32}P end labeled dsDNA probe was performed at room temperature in 20 mM HEPES (pH 7.9), 100 mM KCL, 50 ng Poly dIdC, 4 mM DTT, and protease inhibitors. Binding reactions were electrophoresed on a 5% non-denaturing acrylamide gel.

Conclusion

We suspect that the COX-2 promoter is activated by two fundamentally different processes. First, transcription factors act as modular enhancers to rapidly and transiently activate gene transcription. Second, a more complex activation mechanism is required for prolonged activation or activation that is stimulus and tissue specific. In this second case, activation may be regulated by a highly ordered enhancesome complex that may share some similarities with IFN-β promoter (Briefly reviewed in reference [230]).

APPENDIX A

Caveats of Signaling Experiments

Before considering the specific details of LPS-induced signaling pathways in macrophage, an important factor should be considered. These signaling pathways assume to some extent that murine and human alveolar, peripheral, and peritoneal primary macrophage cells and the plethora of monocytic and macrophage-like cell lines are essentially similar. Since this assumption is not correct, the information discussed here will be limited to what is observed in RAW 264.7 murine macrophage cells when there are conflicts between observations in different cell types. Also important to consider is the method by which these signaling pathways were tested. One general method employs the use of kinase inhibitors. Most of these compounds have a very narrow window of semi-specific activity, and authors often use concentrations that may also non-specifically inhibit other kinases that may be functioning in parallel. This problem is compounded by the over interpretation of results where only partial inhibition of a kinase activity is observed. Another general method of studying signaling networks is to overexpress functional enzymes, constitutively active enzymes, or non-functional enzymes. These dominant active and dominant negative experiments result in hyper-activation of a pathway or blockade of a pathway by out competing the native kinase substrate with that of the non-functional protein. Another problem with adding an active kinase is that it may activate a signaling pathway even though it is not present in the cell under normal conditions. Nevertheless, the conclusions from the data of signaling experiments are the

best approximation of what happens in the cell and should not be discarded out-of-hand and are useful in making inferences to the best possible explanation.

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