June 2000

Regulation of Cyclooxygenase-2 Expression by Lipopolysaccharide and Interleukin-1 in Bone Tissues and in Bone Cells

Anita Bhatt

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REGULATION OF CYCLOOXYGENASE-2
EXPRESSION BY LIPOPOLYSACCHARIDE AND
INTERLEUKIN-1 IN BONE TISSUES
AND IN BONE CELLS

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B.A., Colgate University, 1993
D.M.D., Tufts University, 1997

A Thesis
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Dental Science
at the
University of Connecticut
2000
Master of Dental Science Thesis

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EXPRESSION BY LIPOPOLYSACCHARIDE AND
INTERLEUKIN-1 IN BONE TISSUES
AND IN BONE CELLS

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University of Connecticut
2000
Acknowledgements

I would like to acknowledge my advisory committee: Dr. Pilbeam, Dr. Godwin, and Dr. Nanda for all of their advice and support on this project. I would like to especially thank my major advisor, Dr. Carol Pilbeam, for all the time and effort she put in teaching and guiding me. In addition, I would like to thank all of the generous and friendly people in the laboratory who helped me along the way including Olga Voznesensky, Cindy Alander, Sunil Wadhwa, and Masato Tomita. A special recognition goes out to Amanda Freeman, who taught me many techniques and was always there to answer my questions.

I would like to recognize the faculty and staff of the Orthodontics Department who I have enjoyed working with over the past three years. A very heartfelt thanks goes out to Dr. Nanda for all his teachings and guidance, and for giving me the opportunity to become an orthodontist. I appreciate all that he has done for me both academically and personally. A sincere thank you to Dr Andy Kuhlberg for his academic and clinical expertise and endless support. On the same note, I would like to thank Dr. Johnny Feldman for his time and dedication toward teaching. To my classmates Drs. Derek Priebe, Susan Davis and Jacqueline Sohn as well as past and present residents, I have enjoyed your friendship and support. A special thank you to Jackie Sohn for the daily laughs and companionship.

Finally, I would like to recognize and thank Brent and my parents whose unconditional love, understanding and encouragement have made it possible for me to accomplish everything that I have throughout the years. Thanks for the inspiration.
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Review of Literature

Introduction

Prostaglandins are potent mediators of many physiologic and pathologic processes. In humans, prostaglandins are involved in different functions such as blood clotting, nerve and growth development, wound healing, and immune responses [1]. Inflammatory agents stimulate their production and they may mediate some effects of inflammatory agents on bone. In particular, prostaglandins are potent stimulators of bone resorption [2] and may play a role in the bone loss associated with inflammation. Many studies have shown that prostaglandins, especially PGE$_2$, are involved in the pathogenesis of periodontal diseases [3, 4] and associated with attachment loss [3]. Prostaglandins have also been shown to be involved in orthodontic tooth movement [5] [6]. This project examines regulation of prostaglandins by inflammatory agents in bone cells.

Prostaglandin Metabolic Pathway

Prostaglandin production involves two important regulatory steps. The first step is the release of arachidonic acid from membrane phospholipids by phospholipase A$_2$. The second step is the conversion of arachidonic acid to PGG$_2$ and subsequently to PGH$_2$ (Figure 1). Both reactions are a function of a single enzyme, prostaglandin G/H synthase (PGHS), which acts as both a cyclooxygenase and a peroxidase. Because of its cyclooxygenase functions, the enzyme is commonly called COX. COX is generally the rate-limiting enzyme in the biosynthesis of prostaglandins [7]. PGH$_2$ is converted by
specific isomerases or reductases to PGD$_2$, PGE$_2$, PGF$_{2\alpha}$, prostacyclin (PGI$_2$) and thromboxane A$_2$ (TxA$_2$) [8]. The prostanoids probably exit the cell via facilitated diffusion [8]. Recently, two isozymes of COX have been characterized and labeled COX-1 and COX-2. The COX-1 gene is about 22 kb long with 11 exons, while the COX-2 gene is about 8 kb long with 10 exons [9]. Both enzymes are approximately 60% identical at amino acid and nucleic acid levels and are both about 70 kD in size. COX-1 is constitutively expressed and is thought to be the "housekeeping" enzyme responsible for the production of prostaglandins required to maintain physiologic processes. COX-2 is the inducible form of cyclooxygenase. COX-2 is induced during the inflammatory process and is thought to be largely responsible for the production of prostaglandins involved in pathologic processes. COX-2 is expressed at undetectable levels in most tissues but may be rapidly induced to high levels by numerous factors [7, 8].

**Regulators of Prostaglandin Production**

There are many regulators of bone metabolism that stimulate prostaglandin production in bone. COX-2 expression is stimulated in bone by factors such as cytokines, growth factors, and hormones. These include parathyroid hormone (PTH)[10], transforming growth factors-\(\alpha\) and -\(\beta\)[11], tumor necrosis factor-\(\alpha\)[12], and fluid shear stress[13]. In addition, prostaglandins themselves have been shown to induce COX-2 expression and increase their own production[14, 15]. Interleukin-1 (IL-1) and lipopolysaccharide (LPS) are important inflammatory factors that can stimulate
prostaglandin production by increasing the expression of COX-2 [14, 16]. IL-1 has been shown to induce COX-2 expression and prostaglandin production in cultured neonatal calvariae from mice [12] and in clonal osteoblastic MC3T3-E1 cells which were originally derived from murine calvariae [17]. LPS, a component of the membrane of gram-negative bacteria, can induce COX-2 expression and prostaglandin production in human gingival fibroblasts [18]. Inhibitors of prostaglandin production that act by inhibiting COX-2 expression include IL-4, retinoic acid, and glucocorticoids such as dexamethasone [12, 19].

Much of the regulation of COX-2 production in bone cells is mediated at the level of transcription of DNA into RNA [20-22]. Using osteoblastic clonal MC3T3-E1 murine cells transfected with plasmids carrying sections of the proximal 5'-flanking region of the murine COX-2 gene fused to a luciferase reporter gene, the −371/+70 bp region has been shown to be sufficient to mediate many COX-2 responses, including the response to IL-1 [20, 22]. However, the LPS stimulation of COX-2 expression and of luciferase activity in these cells has not yet been studied.

**Prostaglandins and Bone Resorption**

*Effects on Osteoclast Formation*

Bone is constantly undergoing the process of remodeling in which mineralized bone is resorbed on a particular bony surface, followed by a phase of bone formation. In normal adults, there is a coupling process so that formation follows resorption at each local site. This concept of bone remodeling is based on the hypothesis that bone-resorbing cells or osteoclasts become activated and begin the process of bone resorption.
Osteoclasts have a limited lifespan and undergo apoptosis. Resorption is then followed by a quiescent reversal phase, after which the bone forming cells, appear and make new bone matrix. A number of systemic factors such as PTH and vitamin D₃, and local factors including prostaglandins, leukotrienes, cytokines, and growth factors participate in the bone remodeling process [23]. Many of these factors act on the osteoblasts, which in turn mediate the signals for osteoclastic bone resorption.

Prostaglandins are abundant in bone cells. They are primarily produced and regulated by osteoblasts. Osteoblasts are derived from mesenchymal or stromal stem cells in the bone marrow. The precursor cells differentiate into preosteoblasts and then into mature osteoblasts, which are involved in the synthesis of organic matrix components and mineralization. Osteoblasts have receptors for many factors that regulate bone turnover, such as PTH, IL-1, prostaglandins, estrogens and vitamin D₃. Some osteoblasts (called osteocytes) are incorporated into the mineralized matrix. Osteocytes communicate with each other and with other bone lining cells through cytoplasmic processes. Osteocytes may be the cells that mediate the remodeling processes in response to mechanical loading. Cells of the osteoblast lineage are also involved in the production of paracrine and autocrine factors, such as cytokines and growth factors, which influence bone formation and bone resorption.

Osteoclasts, the bone-resorbing cells, arise from hematopoietic stem cells in the bone marrow. The osteoclast progenitors proliferate and differentiate into mononuclear preosteoclasts and fuse with each other to form multinucleated osteoclasts. The formation of mature osteoclasts requires a contact-dependent interaction between hematopoietic osteoclast progenitors and osteoblasts or other stromal cells (Figure 2).
The molecule responsible for this interaction is a membrane-bound factor that is expressed by osteoblasts or stromal cells in response to most resorption-stimulating factors. This molecule has been recently identified as a receptor activator of nuclear factor (NF)-κB ligand (RANKL), also found on T-cells [25], and is identical to osteoprotegerin ligand (OPGL) [26], TNF-related activation-induced cytokine (TRANCE) [27], and osteoclast-differentiating factor (ODF) [25]. Osteoprotegerin (OPG), also called osteoclastogenesis inhibitory factor (OCIF), is a soluble receptor of the TNF receptor-ligand family and inhibits bone resorption by preventing the binding of RANKL to its receptor RANK [25]. Osteoblastic cells also produce OPG. It was recently reported that OPG inhibits in vitro osteoclast formation from three different signaling pathways stimulated by 1,25(OH)₂D₃, PTH, or IL-11[28].

Prostaglandins have been shown to be necessary for maximal development of osteoclasts in cultures stimulated by IL-1[29], TNFα [30], PTH [31], 1, 25(OH)₂D₃ [32], IL-11[33], and IL-6 [22]. Prostaglandins are thought to enhance osteoclastogenesis largely via increasing RANKL expression and decreasing OPG expression. Prostaglandins as well as 1,25(OH)₂D₃, and PTH have been shown to stimulate bone resorption through the induction of RANKL expression in osteoblasts [34-37]. Yasuda et al. have shown that RANKL gene expression was upregulated in osteoblasts of mice cultured with 1,25(OH)₂D₃, IL-11, PGE2, or PTH [34]. They also demonstrated that OPG inhibited osteoclast formation that was elicited by the same stimulators in cocultures of spleen cells and osteoblasts [34]. Similarly, Lacey et al. reported that in murine bone marrow macrophages, osteoclastic differentiation was increased with treatment of PGE before the addition of osteoblastic/stromal cells, 1,25(OH)₂D₃, and
treatment of PGE before the addition of osteoblastic/stromal cells, 1,25(OH)2D3 and
dexamethasone [38]. Brandstrom et al. showed that PGE2, in a time and dose-dependent
manner, decreased OPG mRNA levels in human bone marrow stroma cells (hBMSC)
[39]. This study also suggests that the down-regulation by PGE2 is mediated via a cAMP
dependent mechanism.

Okada et al. studied tissues from COX-2 knockout mice and showed that the
prostaglandins which enhance osteoclast formation in response to PTH and 1,25 D, are
produced by COX-2 in osteoblasts [40]. They found that in marrow cultures from COX-
2-deficient (-/-) mice, 1,25 D and PTH-stimulated osteoclast formation was decreased
relative to the COX-2 (+/+ ) mice. The decrease was reversed when PGE2 was added to
the cultures. In addition, there was decreased osteoclast formation when the COX-2-
deficient (-/-) osteoblasts were cultured with +/+ or -/- spleen cells, but not when +/+ osteoblasts were cultured with -/- spleen cells or when PGE2 was added to the cultures.
They could therefore conclude that this decreased osteoclast formation is due to the
decreased prostaglandin production by COX-2 in osteoblastic/stromal cells. These same
studies suggest that prostaglandins stimulate osteoclast formation in marrow cultures via
enhanced expression of RANKL.

Studies in spleen cell cultures in which the need for support cells is replaced by
addition of RANKL to the cultures, suggest that prostaglandins also have a direct effect
on osteoclastic precursors to enhance osteoclast formation [32, 40]. The inhibition of
GM-CSF, granulocyte macrophage- colony stimulating factor, an inhibitor of osteoclast
formation may mediate this effect, by prostaglandins [40]. The receptor, which mediates
the effects of PGE2 in the spleen cultures, has been identified as the EP2 receptor (Li and
Macrophage-colony stimulating factor (M-CSF), which is produced by osteoblastic cells, is also necessary for osteoclast development. Osteoblastic cells produce M-CSF constitutively and increase their production in response to proinflammatory agents [41]. Mice in which the M-CSF gene is not functional have osteopetrosis, op/op mice. Takahashi et al. showed that when osteoblastic cells from normal mice were cocultured with spleen cells (osteoclast precursors) derived from op/op mice, osteoclasts were formed [42]. However, osteoclasts never formed in cocultures of normal spleen cells and op/op osteoblastic cells, suggesting that the osteoclast deficiency in these mice is due to a defect in osteoblastic cells but not in spleen cells [42]. The impaired bone resorption in these mice was restored when given M-CSF [43]. These results confirm that M-CSF produced by osteoblasts/stromal cells plays an essential role in osteoclast development. Although M-CSF has been shown to increase osteoclast formation, prostaglandins inhibit M-CSF expression [41, 44]. Thus, the ability of prostaglandins to increase osteoclast formation is probably not through the effects of prostaglandins on M-CSF expression.

Prostaglandin Effects on Resorption in Organ Culture

The earliest studies by Raisz and Martin [45] showed that prostaglandins, especially of the E series (PGE$_2$) stimulated bone resorption in the fetal rat long bone organ culture system through cyclic AMP production. Many factors that stimulate prostaglandin production also stimulate resorption in organ culture. The resorption stimulated by such factors can be mediated in part by prostaglandin production [11, 29,
Previous studies have examined the role of prostaglandin synthesis on bone resorption stimulated by cytokines or other factors. Gowen et al. [47] reported that IL-1 stimulates bone resorption in mouse calvarial cultures via a prostaglandin-independent mechanism. The addition of indomethacin, an inhibitor of both COX-1 and COX-2 activity, did not affect IL-1-induced bone resorption. However, another study using bone organ culture systems found that IL-1 induced bone resorption and osteoclast formation via a prostaglandin-dependent mechanism [29]. Dewhirst et al. also reported IL-1-stimulated resorption in the fetal rat long bone system is blocked by indomethacin, suggesting the involvement of prostaglandins [48]. Although results in organ culture show variable dependence of resorption on prostaglandin production, many studies have found prostaglandins to be necessary for maximal formation of osteoclasts, as noted above. These findings suggest that prostaglandins may play a more important role in the generation of new osteoclasts than in the activity of mature osteoclasts.

Boyce et al. [49] also found both prostaglandin-dependent and prostaglandin-independent stimulation of bone with IL-1 in vivo. They injected mice subcutaneously above the calvaria with IL-1 for 3 days in the presence and absence of systemic indomethacin. The indomethacin was continued after the IL-1 was stopped. During the treatment with IL-1, stimulation of osteoclastic bone resorption was not inhibited by treatment with indomethacin. However, when the IL-1 treatment was stopped after 3 days, the osteoclastic resorption continued up to 27 days and was inhibited by indomethacin [49]. This suggests that the long-term effect was mediated by prostaglandins, consistent with the ability of prostaglandins to stimulate new osteoclast formation.
Prostaglandins and Inflammation

Although the importance of COX activity in the production of prostaglandins has been known for many years, the induction of COX-2 and its role in inflammation has only been appreciated recently [1]. Periodontal disease is an inflammatory disorder that often leads to irreversible alveolar bone resorption and tooth loss. It initiates as a bacterial infection in the gingiva and progresses to attachment loss of the periodontal ligament, which anchors teeth to the surrounding bone. Because there are minimal systemic effects of periodontitis, it is likely that soft tissue and alveolar bone loss around the affected teeth results from the local release of inflammatory mediators secondary to bacterial infection [50]. The bone loss associated with periodontal disease is hypothesized to be a result of an uncoupling of bone resorption and bone formation processes in which local cytokines are released from inflammatory cells. The increased bone loss is thought to reflect an increase in bone resorption compared to bone formation. Some important inflammatory mediators in periodontal disease are PGE$_2$, IL-1, IL-6, and TNF$\alpha$ [51]. Bone cells and resident fibroblasts and leukocytes in the inflammatory infiltrate, especially macrophages, can produce the cytokine mediators. Inhibition of PGE$_2$ production suppresses or blocks alveolar bone destruction in both experimental animals and periodontitis in humans [52-54]. However, the actual functions of these major mediators in normal bone in the periodontium and bone destruction in periodontitis needs further investigation.
Inflammatory Mediators

Interleukin-1 (IL-1):

IL-1, one of the first pro-inflammatory molecules discovered, is a key mediator of the human body's response to microbial invasion, inflammation, autoimmune reactions, and tissue injury [55]. There are two molecular forms of IL-1: IL-1α and IL-1β, which are encoded by separate genes. IL-1 is predominantly produced by macrophages activated by microbial substances, immune complexes or other cytokines, or by lymphocytes. It can also be released from platelets, fibroblasts, keratinocytes, and endothelial cells [56]. IL-1 can also induce its own production[57]. The activity of IL-1 can be suppressed by the release of a specific inhibitor protein, which serves as an IL-1 receptor antagonist (IL-1ra). IL-1ra binds to and occupies the IL-1 receptor, but does not activate the cell receptor. Some of the IL-1 biological effects include cell proliferation, increased collagen and collagenase synthesis, bone resorption, and PGE2 synthesis in fibroblasts [55, 56]. As mentioned earlier, the bone resorbing activity of IL-1 has been studied in vitro and in vivo and some of this activity may be mediated by prostaglandins [46, 49, 58]. IL-1 has been shown to stimulate COX-2 expression in osteoblastic cells and neonatal murine calvaria, thereby increasing prostaglandin production [14, 17]. IL-1 has also induced COX-2 expression in other cell types such as human endothelial cells, human decidual cells, and rat endometrial stromal cells [59-61]. Recently, Nakagawa et al. showed that IL-1α induces COX-2 promoter activity in a dose-dependent manner, and promoted COX-2 mRNA expression in bone-derived endothelial cells suggesting a possible role in bone remodeling. [62].
**Lipopolysaccharides (LPS):**

LPS is one of the main bacterial components responsible for inducing pro-inflammatory cytokine expression, COX-2, and prostaglandin production. It is a critical mediator of the host response in Gram-negative infections. Systemically administered LPS is a potent toxin that can cause fever, shock, intravascular coagulation, and death [51]. LPS is a structural component of all Gram-negative bacterial cell walls, and consists of three structural domains: a lipid A region, a link region, and a repeating carbohydrate side chain. The lipid A region is responsible for many biological responses ranging from fever to bone resorption. The carbohydrate side chain serves as a potent antigen and is responsible for serotype specificity [51]. There are many reports of differing structures of different LPS species from various bacteria; however, the variability in potency and activity of LPS is relatively small considering its vast array of potent biological activities [51]. The major cellular receptor for LPS is CD14, which is present on monocytic cells and neutrophils [63]. There is also a soluble form of CD14 receptor (sCD14) that binds to endothelial cells [63]. LPS must bind to CD14 and a LPS-binding protein to form a complex. The mechanism by which LPS stimulates the cells has not been fully established [64]. When this receptor is occupied, the secretion of pro-inflammatory cytokines such as IL-1, TNF, IL-6, and PGE₂ occurs [51]. Several studies have shown that LPS upregulates expression of the CD14 gene in several kinds of cells [65-67]. Amano et al. recently reported that LPS stimulates endogenous expression of CD14 in murine calvarial cells, and that CD14 is strongly involved in LPS-stimulated bone resorption in these cells [68]. Hence, they suggested a functional role of the CD14 receptor in LPS-stimulated bone resorption.
LPS has been shown to stimulate bone resorption \textit{in vitro} [69-72]. LPS of oral bacteria such as \textit{P. gingivalis}, \textit{A. actinomycetemcomitans}, and \textit{Prev. intermedia} have been implicated in the etiology and pathogenesis of periodontal disease because they can provoke inflammatory and immune responses, and stimulate bone resorption. It has been suggested that LPS can penetrate gingival connective tissue and induce a local inflammatory response that leads to periodontal bone resorption [73]. A recent study by Zubery et al, indicated that live or heat killed \textit{P. gingivalis} stimulates similar bone resorption in mice calvaria [50] and suggests that LPS plays an important role. LPS from periodontopathic bacteria are potent inducers of IL-1[64, 74, 75]. Studies have shown that LPS concentrations as low as 1 ng/ml can activate production, and amounts of IL-1 release have been reported as high as 100 ng/10^6 cells/24 hrs [76].

LPS can stimulate COX-2 expression directly or indirectly. The direct effects are seen early after LPS treatment. Barrios-Rodiles and colleagues reported that LPS-induction of COX-2 mRNA levels, \textit{in vitro}, in human macrophages occurred as early as 2h, peaked at 6-12h and was maintained at high levels at 24 and 48h [77]. They also stimulated macrophages at different times with LPS in the presence or absence of neutralizing anti-IL-1β antibodies. Antibodies to IL-1β did not affect COX-2 mRNA expression induced by LPS at early time points but inhibited the expression of COX-2 after 12h suggesting that the effect of LPS after 12h was mediated by LPS induced IL-1. A recent \textit{in vivo} study examined the effect of LPS on renal COX-2 expression in the rat [78]. This study found that in cultured mouse renal medullary cells, LPS stimulated COX-2 and CD14 expression and the COX-2 expression was reduced by the addition of
CD14 monoclonal antibody, suggesting that LPS stimulated the COX-2 expression through a CD14-dependent mechanism.

Prostaglandin Inhibitors

Nonsteroidal anti-inflammatory drugs (NSAIDS) replace arachidonic acid in the active site of COX enzymes and prevent production of PGH₂ and consequently prostaglandins. NSAIDs inhibit prostaglandin production and are widely prescribed as inhibitors of COX activity to control inflammation and pain. Conventional NSAIDS such as aspirin and indomethacin, nonspecifically inhibit both COX-1 and COX-2 at standard anti-inflammatory doses. The desirable anti-inflammatory and analgesic effects occur through the inhibition of COX-2, but the undesirable effects of gastrointestinal toxicity and bleeding occur as a result of COX-1 inhibition. Recent studies on the effects of NSAIDS, specifically flurbiprofen and ibuprofen, on bone loss in periodontitis revealed a decrease in alveolar bone loss in humans and beagles[79, 80]. The suppression of PGE₂ with these drugs greatly diminishes attachment and bone loss and therefore decreases periodontal disease progression. Other studies have demonstrated a decrease in tooth movement in animal models treated with NSAIDs [5, 81]. Since COX-2 may be the isoform that predominates at sites of inflammation, and COX-1 is responsible for the "housekeeping" functions such as renal blood flow maintenance and gastric cytoprotection, the development of specific COX-2 inhibitors may prove to be more effective than nonspecific NSAIDS in targeting inflammation such as periodontal disease. One such COX-2 specific inhibitor is NS-398, which has recently been introduced.
Futaki et al. reported preferential inhibitory effects of NS-398 on COX-2 activity in rats treated with LPS in vivo [82]. It has also proved to be more potent in inhibiting COX-2 in cultured rat bone cells [83]. A recent study by Shimizu et al. indicated that NS-398 completely inhibited PGE$_2$ synthesis from human PDL cells subjected to mechanical tension force [4].
Hypothesis

We hypothesize that inflammatory agents will transcriptionally induce COX-2 gene expression in bone cells similarly in vivo and in vitro. Specifically, we propose that the inflammatory agents lipopolysaccharide (LPS) and interleukin-1 (IL-1) will induce COX-2 gene expression similarly in (1) freshly isolated calvariae, (2) cultured calvariae, and (3) isolated primary calvarial cells.

Specific Aims

1) To measure COX-2 transcriptional regulation in freshly isolated calvariae from Pluc mice treated in vivo with LPS. We will inject mice with lipopolysaccharide or vehicle and sacrifice mice at varying times up to 8 h after injection. Calvariae will be dissected out and half of each calvarium will be used for measurement of luciferase activity. The other half calvarium will be used to obtain RNA for Northern analysis to determine if COX-2 endogenous gene expression correlates with luciferase activity.

2) To measure COX-2 transcriptional regulation in cultured neonatal calvariae from Pluc mice. Calvarial cultures will be treated with vehicle or LPS or IL-1 for varying times up to 24 h and then extracted for luciferase activity. In some cases, calvariae will also be extracted for RNA to determine endogenous gene expression. RIA to determine COX-2 activity will measure PGE₂ accumulation in the culture media.
3) To measure COX-2 transcriptional regulation in primary osteoblastic cells enzymatically digested from Pluc calvariae. Cultured cells will be treated with varying doses of LPS or IL-1 for varying lengths of time, and cells extracted for luciferase activity. In some cases, RNA will be extracted to determine COX-2 endogenous gene expression. RIA to determine COX-2 activity will measure PGE$_2$ accumulation in the culture media.
**Materials and Methods**

**Materials:** LPS from *Escherichia coli*, 055:B5, for cell culture experiments, dexamethasone (DEX), and Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Sigma Chemicals Co., St. Louis, Mo. LPS from *Escherichia coli*, 026:B6, for tissues, was purchased from Difco Laboratories, Detroit, MI (4mg used for 30g mouse). IL-1α and IL-1β were purchased from Boehringer Manheim, Indianapolis, IN.

**Transgenic Mice:** To further examine COX-2 transcriptional regulation *in vivo*, in organ culture and isolated cells, transgenic mice were developed by the Transgenic Animal Facility (UCHC). The mice used for this study were transgenic for −371/+70 bp of the COX-2 5’-flanking regions fused to a luciferase reporter gene and developed in a CD-1 background. The term “transgenic” refers to the insertion of DNA from any source directly into a mouse through a pronuclear injection technique. The production of transgenic mice involves a specific sequence of events from DNA preparation to DNA analysis by Southern blotting [84]. Once the DNA construct is isolated and purified, it is ready for microinjection. Five-to six-week-old female mice are superovulated, mated to fertile males, and then sacrificed. The DNA construct is microinjected into the pronucleus of a one-cell mouse embryo. The surviving embryos are reimplemented into pseudopregnant foster female mice. When the mice are 3-5 weeks old, they can be screened for the transgenic construct through tail biopsy and DNA analysis by Southern blotting. Mice used in this study are the result either of crossing a male Pluc mouse with
a non-transgenic female mouse (heterozygous) or of crossing two Pluc mice (homozygous). Because the integration site of the DNA construct can affect its expression and regulation, we have developed 4 lines of mice from independently microinjected pronuclei. For each type of experiment, we must confirm that the result is not dependent on the integration site by doing the experiment in different transgenic lines.

**Cell Culture:** All cells were cultured in phenol red free DMEM with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (50 ug/ml) at 37°C in a humidified atmosphere of 5% CO₂ in air.

**Primary osteoblastic (POB) cells:** Calvariae were excised, dissected free of loose connective tissue, washed with phosphate buffered saline (PBS) ph 7.4. and treated with crude collagenase P (Boehringer Mannheim) and trypsin for 10 min at 37°C. The digestion procedure is repeated to provide five populations of cells. Released cells are removed, and the reaction is stopped with DMEM and 10% FCS. We digested neonatal calvariae from a single litter (n=2-8), pooled populations 2-5, and grew cells for 5-7 d to confluence. Confluent cells were split, plated at 5000/cm² in 6-well dishes and cultured for 7 d before treatment with LPS (10µg/ml), IL-1 (2ng/ml), 1µM dexamethasone, and 10 µM arachidonic acid. Cultures were treated for varying time intervals up to 48 h. Luciferase was extracted from n=3 wells for each treatment group and another 3 wells were pooled for RNA extraction.
**Neonatal Calvarial Cultures:** Calvarial cultures permitted us to use less agonist and reduce mice discomfort. Cultured neonatal half-calvariae were treated with LPS (10 μg/ml) and IL-1 (10ng/ml). The matched half calvariae were divided between vehicle (control) and treatment groups. We used 6 half-calvariae in each treatment group, 3 for luciferase activity and 3 for RNA extraction. The induction of COX-2 has previously been shown to be spontaneously induced in the first 24 h by culture in calvariae [85]. Hence, we pre-cultured bones for 24 h before treatment. The cultures were treated for 1.5, 3, 4, 6, 8 and 24 h. Each experiment was repeated 3 times and experiments were repeated to compare responses in 3 different Pluc371 lines.

**Freshly isolated tissues:** Four to six-week old male Pluc371 mice were injected subcutaneously with 4mg of LPS or vehicle. The mice were sacrificed 3-4 h after injection to assess the direct effects of LPS on luciferase activity. Four to six mice were used in each treatment group; the experiments were repeated at least three times. Half of each whole calvarium was taken to measure luciferase activity, and half for RNA extraction. This protocol was repeated for Pluc lines from 3-4 different founders.

**COX-2 Transcriptional Regulation and Luciferase Assay:** The COX-2 gene expression is regulated at the transcriptional level. A typical eukaryotic gene control region consists of a promoter and regulatory sequences. The promoter is the DNA sequence where the general transcription factors and the RNA polymerase assemble. The regulatory sequences are bonding sites for gene regulatory proteins, which control the rate of transcription initiation. These sequences can be located upstream, downstream, or even
adjacent to the promoter, and they can act in combinations to control gene transcription. One way to study the regulation of the promoter region is to fuse it to a reporter gene. A reporter gene encodes a protein that can be easily detected by an enzyme assay or cytological stain. When the recombinant DNA molecules are used to transfect different types of mammalian cells, the level, timing and cell specificity of the reporter gene production will reflect the action of the regulatory DNA sequences in the construct [86].

For the experiments in this study, we use the proximal -371/+70 bp of the 5’-flanking region of the murine COX-2 gene fused to a luciferase reporter gene (Pluc) (Figure 3). The luciferase reporter encodes the firefly (Photinus pyralis) luciferase enzyme, which is used to monitor transcriptional activity. The enzyme catalyzes a chemiluminescent reaction that produces a flash of light. The light production is measured using a luciferase assay and luminometer. The -371/+70 bp of the 5’-flanking region was chosen because previous studies have shown that for most agonists studied, the major cis-acting sites for induction of luciferase activity lies between -150/+70 and -40/+70 bp [20, 21].

Previous studies in our lab examined transcriptional regulation of COX-2 expression in MC3T3-E1 cells stably transfected with COX-2 promoter-luciferase reporter (Pluc) constructs. These studies showed that the stimulation of luciferase activity is associated with the induction of COX-2 expression [10, 20, 87, 88].

(1) Cells. Luciferase activity was measured in soluble cell extracts prepared with a kit from Promega (Madison, WI) using an automatic injection luminometer (Berthold Lumat; Wallac Inc., Gaithersbury, MD). For each experiment, 3 wells of a 6-well dish of cells were analyzed per treatment group. Cells were scraped in 200 µl of lysis buffer, centrifuged for 30 sec and supernatants collected. 100 µl of substrate is injected into 10-
20 μl of supernatant and counts per second counted for 10 sec. Activity was normalized to total protein measured with a bicinchoninic acid (BCA) protein assay kit (Pierce; Rockford, IL). Samples were mixed with BCA on a microtiter plate and incubated at 37°C for 30 minutes. Absorbance was read at 562 nm with a microtiter plate reader (Dynatech MR5000, Dynatech Laboratories) and the protein concentration of the samples was determined.

(2) Tissues. Tissues were placed in 500 μl of lysis buffer and polytroned for 20-30 seconds. The extracts were centrifuged for 2 minutes and the supernatants collected and frozen overnight at -20°C. The supernatants were assayed the next day.

**Northern Blot Analysis:** Total RNA was extracted by the method of Chomczynski and Sacchi [89]. Three 35mm wells of POBs cells and 3-6 half calvariae were used. Cells or tissues were washed with phosphate buffered saline (PBS) and homogenized in 4M guanidinium thiocyanate (GTC) by polytron, extracted with phenol/ chloroform-isooamyl alcohol (24:1), and RNA precipitated with isopropanol and washed with 80% ethanol. After quantitation at 260nm, 10-20 μg of total RNA was prepared with ethidium bromide to run a gel. The samples of total RNA are run on a 1% agarose-2.2 M formaldehyde gel and transferred to a nylon membrane by positive pressure. The RNA is fixed to the membrane by UV irradiation using a Stratagene UV Stratalinker 1800. After 3 h of prehybridization in a 50% formamide solution at 42°C, filters were hybridized overnight in a similar solution in rotating cylinders at the same temperature with the random primer [³²P] dCTP-labeled cDNA probe. Filters were washed once in a 1X SSC, 1% SDS solution at room temperature, once in 0.1X SSC, 0.1% SDS solution at 65°C, and then 4
more times in the latter solution at room temperature. After washing, the filter was
exposed to Kodak XAR-5 film at -70°C. Filters were hybridized sequentially with
cDNAs for COX-2, luciferase (LUC), and a housekeeping gene, glyceraldehyde-3-
phosphate dehydrogenase (GAPDH). Filters were stripped with boiling 0.1% SDS and
0.1 X SSC between hybridizations. Signals were quantitated by densitometry.
Autoradiographs were scanned and analyzed using NIH Image software.

**PGE$_2$ Assays:** Medium was removed from cultured calvariae or POB cells and PGE$_2$
accumulation was measured as described by Raisz and Simmons[90]. \(^{3}\text{H}\)-
prostaglandins are used as a tracer and the assays run at antibody dilutions providing 20
to 40% binding. The lower detection limit of the assay for PGE$_2$ is approximately 5 pg
per tube or 0.14 nM in a 100μl sample, and the intra assay variation was 6%. The assays
are carried out at 4°C and free prostaglandin removed with dextran-coated charcoal. The
values for unknowns were calculated from a standard curve using the logit-log, curve-
fitting computer program (simple RIA program, D. Rodbard, BCTIC, Computer Code
Collection, Nashville, TN). Data is presented as nM.

**Statistical Analysis:** Statistical significance of differences was determined by analysis of
the variance (ANOVA) with post-hoc comparison by the Bonferroni method or ANOVA
by Ranks.
Results

**Effects of LPS and IL-1 on COX-2 expression and luciferase activity in cultured calvariae from transgenic mice:**

We have previously shown that COX-2 mRNA is not detectable in freshly isolated calvariae. However, COX-2 mRNA is rapidly and transiently induced on culturing the calvariae [85]. Hence, we “pre-cultured” the calvariae for 24 h before treatment with agonists to allow this initial induction to dissipate. Cultures were then treated with vehicle or LPS (10 µg/ml) for 0, 4, 6, 8, and 24 h. COX-2 mRNA levels were determined by Northern analysis. COX-2 mRNA levels were increased at 4 h compared to 0 h in vehicle treated cultures and gradually decreased thereafter (Fig 4). LPS increased COX-2 mRNA levels relative to controls at 4, 6 and 8 h and levels remained slightly elevated at 24 h. The peak effect of LPS was seen at 4 h.

In the same culture system, our lab has previously shown that IL-1 (10 ng/ml) stimulated COX-2 mRNA expression in cultured neonatal mouse calvariae [14]. The peak effect of IL-1 was at 4h and effects were maintained at 24 h.

To determine if the LPS induction of COX-2 mRNA was transcriptionally mediated, we measured luciferase activity in cultured calvariae. Because there was a possibility that the expression of the transgene would vary depending on the site of chromosomal DNA incorporation, we compared LPS (10 µg/ml)-stimulated luciferase activity in calvariae from different lines of transgenic mice. Because there was peak expression of COX-2 mRNA levels at 4 h, we chose a time point of 6 h to allow for the production of the functional luciferase enzyme. Table 1 shows a comparison of the mean
luciferase activity, in counts per second (cps), normalized to total protein (μg), and fold-increases in four experiments. The basal Pluc activity was lowest in Line 157 (1.0 ± 0.1), highest in Line 145 (143.6 ± 13.9) and intermediate in Line 143 (11.1 ± 2.9, 13.2 ± 2.7). Although the basal levels of Lines 157 and 145 were markedly different, the fold-increases (treated/control ratio) with LPS treatment, 4.8 and 3.8 respectively, were similar. Comparison of two experiments using Line 143 showed similar basal activities but the fold-increases were 2.2 and 7.8. However, for Line 143, the LPS effect on luciferase activity was not statistically significant in the experiment with the fold-increase of 7.8. Including only the statistically significant results, the effect of LPS on luciferase activity was 2-5 fold for all lines.

We also examined the IL-1 effects on luciferase activity in cultured calvariae from two lines of transgenic mice at 6 h. Figure 5 compares the effects of IL-1 (2ng/ml) and LPS (10 μg/ml). IL-1 increased the mean luciferase activity significantly by 2.6-fold (Line 143) and 4.9-fold (Line 145) after 6h of treatment. This was similar to the fold-increases seen with LPS.

To determine if the medium PGE₂ levels corresponded with the LPS and IL-1 stimulated luciferase activity, we measured PGE₂ production by RIA in the same experiments shown in Fig 5. After 6 h, the basal (control) levels of PGE₂ were similar for lines 143 (6.7 ± 1.2 nM) and 145 (7.0 ± 2.1 nM) (Table 2). The levels were much higher after treatment with LPS. There was a 6.2 fold-increase in Line 143 and a 15.1 fold-increase in Line 145 (Table 3).

The LPS-stimulated increase in PGE₂ production was 3-4 fold greater than the LPS- stimulated increase in Pluc activity. This discrepancy between luciferase activity
and PGE$_2$ production suggests that LPS regulates COX-2 through other promoter regions than in our Pluc construct or that LPS regulates arachidonate availability.

There were fewer differences seen with IL-1 treatment in the same experiment. In Lines 143 and 145, IL-1 stimulated both Pluc activity and PGE$_2$ production 3-5 fold, indicating that the PGE$_2$ response to IL-1 is largely the result of transcriptional induction of COX-2 via the $-371/+70$ bp flanking region.

*Effects of dexamethasone (Dex) on cultured calvariae from transgenic mice:*

To determine the effect of dexamethasone (Dex) on endogenous expression of COX-2, we treated cultured neonatal mice calvariae (Line 145) with vehicle (control), IL-1 (2 ng/ml), Dex ($10^{-6}$M), and Dex + IL-1 for 6 h. In this experiment, we saw expression of COX-2 mRNA in control cultures after 6 h (Fig 6). The COX-2 mRNA level was increased after IL-1 treatment. Dex inhibited COX-2 mRNA expression in both control cultures and IL-1 treated cultures. With Dex + IL-1 treated calvariae, COX-2 expression was detectable, but to a much less extent than in IL-1-treated and control cultures.

We also examined luciferase activity in the same experiment as shown in Figure 6 to determine the effect of Dex. After 6 h, control levels of mean luciferase activity were $4.4 \pm 0.8$ cps/µg protein. There was an increase after IL-1 treatment ($198.7 \pm 131.5$ cps/µg protein) (Fig 7). Because there was a large SEM for the IL-1 treated cultures, there was no statistically significant effect of IL-1. Luciferase activity in Dex and Dex + IL-1 cultures was $32.4 \pm 9.1$ cps/µg protein and $42.1 \pm 23.2$ cps/µg protein, respectively, suggesting that Dex inhibited IL-1 stimulated luciferase activity. We also measured the medium PGE$_2$ levels in this experiment. Control and IL-1 cultures had PGE$_2$ levels of
58.6 ± 23.2 nM and 84.1 ± 51.5 nM, respectively. The IL-1 treated groups had a high SEM in and therefore; the differences from control were not significant. The levels for the Dex and Dex + IL-1 groups were 10.6 ± 0.8 nM and 22.7 ± 7.6 nM, respectively. Due to the large SEM, we repeated this experiment in a different line (Line 157) and measured luciferase activity at 6h (Fig 8). Although the trend for an increase in luciferase activity in IL-1 treated cultures and a decrease in luciferase activity in Dex + IL-1 treated cultures was the same as seen in the previous experiment, the differences were not statistically significant.

*Effects of LPS and IL-1 on luciferase activity in primary osteoblastic cells derived from transgenic mice:*

To compare the LPS and IL-1 induction of COX-2 expression in cultured calvaria and primary osteoblastic cells, we measured luciferase activity in primary osteoblastic cells derived from neonatal transgenic mouse calvariae. Figure 9 shows a comparison of effects of the IL-1 and LPS on luciferase activity in primary osteoblastic cells from Line 157. Cells were treated with vehicle (Control), LPS (10 μg/ml) and IL-1 (2 ng/ml) for 0, 3, 4.5, 6, 7.5, and 24 h. Peak expression of luciferase activity was seen between 4.5-6 hours for both LPS and IL-1 treated cells. Table 4 shows a comparison of the mean luciferase activity, in counts per second (cps), normalized to total protein (μg), and fold-increases in six experiments. The cells were treated with vehicle (control) or LPS (10 μg/ml) for 6 h. Basal activities for the four different lines ranged from 6.0 to 30.5 cps/μg protein, except for one experiment with line 157 where the basal activity was 305.4 cps/μg protein. Despite differences in the basal activities, LPS stimulated a 2 to 4 fold-
increase in luciferase activity for all lines. We also compared IL-1 (2 ng/ml)-stimulated luciferase activity in primary osteoblastic cells derived from neonatal transgenic mice calvariae (Table 4). IL-1 induced luciferase activity significantly by 2-4 fold for all lines. Both IL-1 and LPS had similar effects on luciferase activity in primary osteoblastic cells and cultured calvariae from transgenic mice.

In another experiment, we measured medium PGE₂ levels (nM) to determine if prostaglandin production corresponded with luciferase activity (cps/µg protein) in primary osteoblastic cells derived from transgenic mice calvariae (Line 157) (Fig 10). Cells were treated with vehicle, LPS (10 µg/ml), or IL-1 (2 ng/ml) for 6 h, with and without arachidonate (AA) (10⁻⁵ M). The arachidonate was added to provide excess substrate for COX, allowing maximal COX (both COX-1 and COX-2) activity to be determined. LPS increased luciferase activity 2.3 fold (treated/control) both without AA and with AA at 6 hours. IL-1 induced luciferase activity 4.1 fold without AA and 3.2 fold with AA. At 6 h, the medium PGE₂ in control cultures was 3.9 nM and 6.4 nM without and with AA, respectively. LPS increased PGE₂ levels by 2.5 fold without AA and 9.5 fold with AA. IL-1-stimulated PGE₂ production exhibited similar fold-increases both with and without AA, (2.0 and 2.8 fold, respectively). Hence, for IL-1, the maximal increase in PGE₂ concentration obtained in the presence of AA to provide excess substrate, correlated with the increase in luciferase activity. For LPS, however, the maximal increase in PGE₂ concentration was 3x greater than the increase in luciferase activity.

We also conducted an experiment using indomethacin (Indo) to determine if endogenously produced PGs mediated some of the increase in Pluc activity stimulated by
LPS in Line 145 (Fig 11). In this experiment, primary osteoblastic cells were treated with vehicle (control), LPS (10 μg/ml), Indo (10⁻⁶ M) or Indo + LPS for 6 h. LPS significantly increased Pluc activity to 73.3 ± 6.5 and 119.5 ± 7.7 cps/μg protein, without and with Indo, respectively. Hence, Indo did not decrease LPS-stimulated Pluc activity.

**Effects of LPS on COX-2 Expression and luciferase activity in freshly isolated tissues from transgenic mice:**

COX-2 mRNA levels were determined by Northern analysis in freshly isolated calvariae from an 11.5 week old transgenic mouse (Line 145) (Fig. 12). The mouse was injected with 4mg of LPS or with vehicle, and sacrificed after 3.5 h. Expression of COX-2 mRNA and luciferase (Luc) mRNA was not detectable in vehicle treated mice. Both COX-2 mRNA and Luc mRNA levels were highly detectable after 3.5 h in the LPS treated mice.

In another experiment and different line (Line 128), LPS induced COX-2 expression in one half of freshly isolated mouse calvaria after 3.5 h (Fig. 13). Again, LPS markedly increased COX-2 mRNA levels relative to control.

To determine if the LPS induction of COX-2 mRNA was transcriptionally mediated *in vivo*, we measured luciferase activity in the other half of the freshly isolated calvariae used for RNA in the experiment shown in Fig 13. Figure 14 shows measured luciferase activity in counts per second (cps), normalized to total protein (μg). LPS increased luciferase activity from 128 to 863 cps/μg protein after 3.5 h, in an increase of 7-fold. The COX-2 mRNA was induced to 2.6 with LPS treatment. Hence, there was an induction of COX-2 mRNA and luciferase activity *in vivo* as seen *in vitro*. 
We also examined LPS-stimulated fold increases (treated/control) in luciferase activity (cps/µg protein) for different freshly isolated tissues. Figure 15 illustrates the varying fold-increases of several tissues from 3-5 independent experiments. Calvarial bone had the highest expression of luciferase activity upon LPS stimulation, exhibiting a 20-fold increase. Lung, kidney, heart and colon had 5-12-fold increases and brain had the lowest fold-increase in luciferase activity (2-fold).
Discussion

Many previous studies of COX-2 transcriptional regulation have used clonal MC3T3-E1 cells, an immortalized osteoblastic cell line derived from murine calvariae that were transfected with the COX-2 promoter-luciferase reporter (Pluc) construct [20, 87, 91]. The use of immortalized osteoblasts for studying transcriptional regulation has been criticized as not being representative of more ‘normal’ osteoblastic cells. Also, Krebsbach et al. found differences in COL1A1 promoter activity between primary bone cells and cultured calvariae from neonatal transgenic mice, suggesting that regulation was changed by taking cells out of their normal matrix environment [92]. Hence, the rationale for using the different systems such as cultured calvariae, primary osteoblastic cells, and in vivo tissues is to (1) compare the COX-2 transcriptional regulation in ‘normal’ osteoblasts from mice transgenic for the Pluc constructs with previous results from MC3T3-E1 cells, and (2) to compare regulation in bone tissue with regulation in bone cells. In vivo studies require large number of mice and agonists and cause mice discomfort. Therefore, if in vivo regulation is similar to that seen with primary osteoblasts, then this latter model would be used for further transcriptional studies. We wanted to examine the effects of the inflammatory agents, IL-1 and LPS, on COX-2 regulation. Because in vivo injections of IL-1 are costly, we chose to use LPS for in vivo studies.

Overall, we found the regulation of COX-2 expression and Pluc activity in cultured neonatal calvariae, primary osteoblastic cells, and in vivo mice experiments to be similar. COX-2 mRNA levels were low or undetectable on Northern analysis under
unstimulated conditions. However, luciferase activity was measureable under the same conditions. This may be attributed to the fact that COX-2 is continuously being transcribed and rapidly degraded such that it is low or undetectable on the Northern analysis.

LPS induced COX-2 mRNA expression similarly for both cultured and freshly isolated calvariae after 3.5-4 h. Northern analysis of cultured calvariae revealed a transient expression of COX-2 mRNA with peak expression at 4 h and decreasing levels by 24 h. LPS induced Pluc activity similarly (2-5 fold) in vitro for different lines of cultured calvariae and primary osteoblastic cells with a peak response at 6 h. Peak expression in luciferase activity was seen two hours later than the COX-2 mRNA expression possibly because of the time needed to allow for the production of the luciferase enzyme.

IL-1 had similar effects on both Pluc activity and COX-2 mRNA expression in cultured calvariae. For both cultured calvariae and primary osteoblastic cells, the IL-1 induction of Pluc activity was 2-5 fold, which parallels the treatment with LPS. Moreover, the IL-1 induction of COX-2 mRNA and of luciferase activity in primary osteoblast cells paralleled the induction in MC3T3-E1 cells seen in previous studies [20].

To determine if the changes in PGE₂ production could be largely explained by transcriptional induction of COX-2, we measured PGE₂ levels in the medium of calvarial cultures and compared them with Pluc activity. In primary osteoblastic cells, IL-1 stimulated Pluc activity by 3-4 fold and increased PGE₂ by 2-3 fold. The similar increase in Pluc activity and PGE₂ production with IL-1 suggests that most of the IL-1 stimulated PGE₂ production occurs via IL-1 inducing new COX-2 gene expression.
In calvarial cultures, the PGE\textsubscript{2} production stimulated by LPS was 3-4 times greater than luciferase activity stimulated by LPS. This could be explained by the fact that LPS increased arachidonate availability or that promoter regions (putative sites) other than those in the Pluc construct are involved. We added excess arachidonate to osteoblast cultures so that substrated release would not be limiting. Nevertheless, LPS stimulated PGE\textsubscript{2} production was 3-fold greater than LPS stimulated luciferase activity. It is possible that LPS increases COX-1 activity, although we did not see an increase in COX-1 mRNA with LPS (data not shown). It is also possible that regions of the COX-2 promoter not in our $-371/+70$ bp construct mediate some of the effects of LPS, and we can assess this possibility by studying larger promoter constructs.

Glucocorticoids such as cortisol and dexamethasone have been shown to inhibit the IL-1 or LPS- induction of COX-2 in various cells including human monocytes, macrophages, and cultured mouse calvarial cells [14, 93, 94]. We found that dexamethasone ($10^{-6}$ M) inhibited the IL-1 induction of COX-2 mRNA. Although the data suggested that dexamethasone also inhibited luciferase activity and PGE\textsubscript{2} production, the differences were not statistically significant. Therefore, we can not conclude that effects of dexamethasone on COX-2 are transcriptional. Although we did not measure the effects of dexamethasone on COX-2 \textit{in vivo}, further investigation \textit{in vivo} would be necessary to determine if the LPS and IL-1-induction of COX-2 activity is inhibited by dexamethasone similarly \textit{in vitro} and \textit{in vivo}.

In \textit{in vivo} studies, the LPS-stimulated COX-2 mRNA expression correlated with Pluc activity for calvarial bone suggesting that similar regulation of COX-2 and Pluc occurred \textit{in vivo} and \textit{in vitro}. Preliminary studies in our lab found that mouse calvarial
bone was among the tissues that most highly expressed COX-2 after LPS (4mg) injection. The other tissues compared were heart, lung, colon, spleen, kidney, liver and brain. The lowest fold induction of luciferase activity was seen in brain; however, this did not correlate with the Northern analysis in which there was a marked increase in LPS-stimulated COX-2 mRNA expression (data not shown). One possible explanation for this discrepancy is that there are missing regions from the −371/+70 bp COX-2 promoter resulting in decreased induction of the transgene. It is also possible that LPS may be regulating post-transcriptional regulation of COX-2 mRNA which may increase COX-2 mRNA expression.

We found a great deal of variability in luciferase activity stimulated by LPS for the tissues studied in vivo. Comparison of the same tissue from different mice could vary because of different rates of LPS metabolism in different mice, differences in injection times or sites, and differences in the exact location of tissues sampled. In addition, there may be differences between the chromosomal integration sites of mice affecting the regulation of the Pluc construct but we did not see large differences in our in vitro studies of calvariae and osteoblastic cells.

In summary, our study suggests that in calvarial cultures and freshly isolated calvariae, the LPS induction of luciferase activity paralleled the LPS induction of COX-2 mRNA. IL-1 (2ng/ml) and LPS (10 μg/ml) stimulated luciferase activity similarly in calvarial and primary osteoblastic cell cultures. Furthermore, IL-1 increased both PGE₂ production and luciferase activity similarly. LPS increased PGE₂ production more than luciferase activity. We can conclude that the COX-2 response to IL-1 is largely due to
transcriptional induction via the −371/+70 bp region of the COX-2 promoter. The maximal response to LPS may require additional regions of the COX-2 promoter.
Tables
Significantly different from control, p < 0.05.

Cultures were treated with vehicle (Control) or 10 ng/ml (for 6 h). Data are means ± SEM for 4-6

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Table I. Comparison of luciferase activity (cpsi/mg protein) in cultured calvariae from different lines of transgenic mice.
Cultures were treated with vehicle (control), LPS (10 ng/ml) or IL-1 (2 ng/ml) for 6 h. RIA was performed as described in Materials and Methods. Data are means ± SEM for ≥4 measurements. * Significantly different from control, p > 0.05. ** Significantly different from control, p < 0.05 as determined by ANOVA on ranks.

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Table 2. Comparison of medium PGE2 (μg/mg DNA) in cultured calvariae from two different lines of transgenic mice.
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&ure 3. Comparison of fold-increases in luciferase activity (cpm/μg protein) and medium PGE2 (μM) in cultured calvariae from two different lines of transgenic mice.

I. Line 145

#14280

I. Line 143

#14280
Data are means ± SEM for 3-6 measurements. *Significantly different from control, p < 0.05.

Cells were treated with vehicle (control), LPS (10 ng/ml) or IL-1 (2 ng/ml) for 6 h.

<table>
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osteoblastic cells derived from four lines of transgenic mice.

Table 4: Comparison of iNOS expression (mRNA) in primary
Figures

Figure 1: Pathway of prostaglandin production. Arachidonic acid is released from cellular membranes by regulated phospholipases, converted to PGH₂ via COX, and then PGH₂ is converted to PGs by individual PG synthases. The two COX isoforms, COX-1 and COX-2, are approximately 60% identical at nucleic and amino acid levels.

Figure 2: Contact-dependent interaction between hematopoietic osteoclast progenitors and osteoblasts or other stromal cells for the formation of mature osteoclasts. The interaction of RANK with RANK ligand is required for differentiation into mature, bone-resorbing cells.

Figure 3: The COX-2 promoter-luciferase reporter construct (Pluc). The -371/+70bp of 5' flanking region of the COX-2 promoter was fused to a luciferase reporter gene.

Figure 4: Lipopolysaccharide (LPS) induction of COX-2 mRNA levels in cultured calvariae from transgenic mice (Line 143). Cultures were treated with vehicle or LPS (10 µg/ml) for times indicated. RNA was extracted and Northern analysis was performed as described in Materials and Methods.

Figure 5: Comparison of IL-1 and LPS-stimulated luciferase activity in cultured calvariae from two lines of transgenic mice. Luciferase activity, in counts per second (cps), was normalized to total protein. Cultures were treated with vehicle (Control), LPS (10 µg/ml) or IL-1 (2ng/ml) for 6 h. Fold activity was calculated as treated/control ratios. Data are means ± SEM for 3-4 wells. *Significantly different from control, p < 0.01.
Figure 6: Inhibition of COX-2 mRNA expression by dexamethasone (DEX, $10^{-6}$ M) in cultured calvariae (Line 145). Cultures were treated for 6 h. RNA was extracted and Northern analysis was performed.

Figure 7: Comparison of luciferase activity and PGE$_2$ levels in cultured calvariae from Line 145. Cultures were treated with vehicle (Con), IL-1 (2 ng/ml), Dex ($10^{-6}$ M) or Dex + IL-1 for 6 h. Luciferase activity, in counts per second (cps) was normalized to total protein. PGE2 concentration was calculated as nM. Bars are means ± SEM for 3 measurements.

Figure 8: Luciferase activity (cps/μg protein) in cultured calvariae from Line 157. Cultures were treated with vehicle (Con), IL-1 (2 ng/ml), Dex ($10^{-6}$ M) or Dex + IL-1 for 6 h. Bars are means ± SEM for 4-5 measurements.

Figure 9: Comparison of luciferase activity (cps/μg protein) in primary osteoblastic cells from Line 157. Cells were treated with vehicle (Control), LPS (10 μg/ml), or IL-1 (2 ng/ml) for 0, 3, 4.5, 6, 7.5, and 24 h. Peak expression was seen between 4.5-6 h. Data are means ± SEM for 3 measurements.

Figure 10: Comparison of luciferase activity and PGE$_2$ concentration in primary osteoblastic cells derived from neonatal transgenic mice calvariae. Cells were treated with vehicle (Con), LPS (10 μg/ml) or IL-1 (2 ng/ml) for 6 h with and without arachidonate ($10^{-5}$ M). PGE$_2$ levels were measured by RIA. Numbers above bars indicate fold-increases (treated/control ratios). Bars are means ± SEM for 3-6 measurements. * Significantly different from control, p < 0.05 as determined by ANOVA. ** Significantly different from control, p < 0.05 as determined by ANOVA on RANKS.
Figure 11: Comparison of luciferase activity (cps/μg protein) in primary osteoblastic cells from Line 145. Cells were treated with vehicle (Control), LPS (10 μg/ml), Indo (10^{-6} M) or Indo + LPS for 6 h. Bars are means ± SEM for 3-6 measurements.

Figure 12: Northern analysis of COX-2 mRNA and Luciferase (Luc) mRNA levels in freshly isolated calvaria from an 11.5 week old transgenic mouse (Line 145). The mouse was injected *in vivo* with vehicle (-) or 4 mg of LPS (+) and sacrificed 3.5 h after injection.

Figure 13: Northern analysis of COX-2 mRNA levels in freshly isolated calvariae from transgenic mice (Line 128). Mice were injected *in vivo* with vehicle (-) or 4 mg of LPS (+). Mice were sacrificed 3.5 h after injection. GAPDH mRNA and 18 S and 28 S ribosomal RNA on gel are shown to assess RNA loading.

Figure 14: Comparison of COX-2 mRNA normalized to GAPDH mRNA and luciferase activity in freshly isolated calvariae from transgenic mice (Line 128). Mice were injected *in vivo* with vehicle or 4 mg of LPS. Mice were sacrificed 3.5 h after injection.

Figure 15: Comparison of LPS-stimulated fold-increase in luciferase activity in different tissues. Mice were injected *in vivo* with vehicle or 4 mg of LPS and sacrificed 3-4 h later. Bars are means ± SEM from 3-5 independent experiments.
**Cyclooxygenases**

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<th>COX-1</th>
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Figure 4

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![Image of gel with COX-2 and GAPDH bands](image-url)
Figure 6

Control  IL-1  Dex  Dex+IL-1

COX-2 mRNA
GAPDH mRNA
Figure 7

PEG2 Concentration

Luciferase Activity

cps/lge protein

Con

IL-1

dex

IL-1

dex + IL-1

Con

(ng/ml)
Figure 8

Luciferase Activity (cps/µg protein)
Figure 9

Luciferase Activity (cps/μg protein)

- Control
- LPS
- IL-1

Time (hr)
Figure 11

Luciferase Activity (cps/μg protein)
Figure 12

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<tr>
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<td>-</td>
<td>+</td>
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Figure 13

LPS
-  +

COX-2

GAPDH

28S
18S
Figure 15
References


synthase-2 mRNA and promoter activity in MC3T3-E1 osteoblastic cells.


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