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Feedback regulation of Cyclooxygenase-2 transcription *ex vivo* and *in vivo*

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Abstract

Cyclooxygenase-2 (COX-2) is a rate-limiting enzyme for prostaglandin biosynthesis. Its inducible expression is regulated by complex pathways.

To monitor *Cox-2* transcriptional activity *in vivo*, we generated a knock-in mouse expressing a firefly luciferase reporter. In this study we examined, by comparing luciferase activity of *Cox-2^{luc/+}* and *Cox-2^{luc/-}* cells and mice, effects of prostanoid products on *Cox-2* promoter transcriptional activation. In peritoneal macrophages, luciferase induction by LPS in *Cox-2^{luc/-}* cells was less than that of *Cox-2^{luc/+}* cells. However, in the presence of PGE₂, induction was comparable, suggesting positive *Cox-2* feedback regulation by PGE₂ occurs for macrophages. In contrast, feedback modulation was not observed in TPA-induced *Cox-2^{luc/+}* and *Cox-2^{luc/-}* mouse embryonic fibroblasts (MEFs). Using non-invasive *in vivo* imaging, we observed negative feedback regulation of *Cox-2* expression during paw inflammation in living mice. Our results suggest *Cox-2* expression is regulated by cell type specific feedback mechanisms, both in cultured cells and in living animals.

Keywords

prostaglandin; peritoneal macrophage; embryonic fibroblast; *in vivo* imaging; inflammation

Introduction

The cyclooxygenases (COXs) catalyze the conversion of arachidonic acid to PGH₂, the common intermediate in prostaglandin, prostacyclin and thromboxane synthesis. Expression of the constitutive *Cox-1* gene is regulated by a GC-rich “housekeeping” promoter lacking TATA or CAAT box sequences [1,2]. The *Cox-2* gene is an immediate-early gene whose transcription is induced by a variety of ligands, including tumor promoters, growth factors, cytokines, endotoxins and mitogens, in a variety of cells [3,4].

A number of signal transduction pathways and transcription factors mediate *Cox-2* induction; moreover, signal transduction pathways for *Cox-2* induction vary depending on stimulus and cell type [5]. The cyclic AMP responsive element (CRE), nuclear factor-interleukin 6 elements (NF-IL6), and an NF-κB element were characterized initially as key transcriptional regulatory sequences in the *Cox-2* promoter [6,7]. A peroxisome proliferator response element (PPRE),

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sterol response element, NFAT element, PU.1/ets binding site and erb-B2/HER2 binding site were also identified as functional *Cox-2* promoter elements [5,8-10]. In addition to transcriptional regulation, COX-2 expression is also regulated by post-transcriptional message stabilization and by regulation of *Cox-2* message translation [11,12].

Prostanoids are reported to feedback regulate *Cox-2* gene expression. However, prostanoid feedback regulation of *Cox-2* gene expression is dependent both on cell type and prostanoid product [13-17]. Moreover, COX-2 is upregulated in a tissue-specific fashion in *Cox-1* knockout mice, indicating that compensatory mechanisms regulate prostaglandin production differently in alternative tissues [19,20]. These studies suggest that COX-2 expression is adjusted, in a tissue/cell type specific manner, by the prostaglandins whose synthesis it mediates.

To investigate *Cox-2* gene expression and to understand the role of this gene in the context of the living animal, we generated knock-in mice in which firefly luciferase is expressed at the translation start site of the endogenous *Cox-2* gene [21]. The *Cox-2* luciferase knock-in allele is also a functional *Cox-2* knockout allele. Loss of functional COX-2 expression from the *Cox-2^{luc}* allele provides a tool to investigate whether prostanoid products resulting from *Cox-2* gene expression affect subsequent *Cox-2* promoter transcription. Here we use *Cox-2^{luc/+}* and *Cox-2^{luc/-}* macrophage and fibroblasts to examine effects of COX-2 regulated prostanoids on subsequent *Cox-2* transcription in culture. We also address *Cox-2* feedback regulation in living animals, using *in vivo* optical imaging of *Cox-2^{luc}* mice.

Materials and methods

Mice carrying the *Cox-2* luciferase and knockout alleles were described previously [21]. LPS (Lipopolysaccharide; *Escherichia coli* serotype 0111:B4), Zymosan and TPA (tetradecanoylphorbol acetate) were from Sigma (MO, USA). PGE₂, PGD₂ and NS398 were from Cayman Chemicals (Ann Arbor, MI, USA).

Macrophages were isolated by peritoneal lavage three days after peritoneal injection (three ml) of 3% thioglycolate medium (Sigma). Cells were washed, resuspended and plated in DMEM/10% FBS. Fibroblasts from *Cox-2^{luc/+}* and *Cox-2^{luc/-}* embryos (12.5 dpc) were prepared as described [21]. LPS zymosan, TPA, PGE₂, and PGD₂ treated cells were assayed for luciferase activity as described [21].

Inflammation was induced by intraplantar zymosan (2.0% in PBS, 30 µl) injection into the right hind paw, as described [22]. The left hind paw received PBS. For *in vivo* imaging, mice were anesthetized, injected with luciferin, and imaged with an IVIS imaging system (Xenogen) as described [21].

Results

To investigate whether COX-2 protein, or any of the prostanoid products resulting from COX-2 induction, affect transcriptional activation of the *Cox-2* gene we used mice carrying a *Cox-2^{luc}* knock-in allele [21]. Because the *Cox-2^{luc}* allele is also a *Cox-2* knockout allele, luciferase activity of *Cox-2^{luc/-}* cells or mice reflects *Cox-2* promoter transcriptional activity in the absence of COX-2 protein or any of its downstream products.

Macrophages generate prostaglandins in response to endotoxin. However, peritoneal macrophages from *Cox-2^{-/-}* homozygous knockout mice completely lack endotoxin-induced PGE₂ synthesis [23]. *Cox-2^{luc/-}* mice are homozygous for their inability to express functional COX-2 and should, therefore, similarly not produce PGE₂. *Cox-2^{luc/+}* luciferase knock-in mice were crossed with *Cox-2^{+/-}* mice and peritoneal macrophages were isolated from *Cox-2^{luc/+}*

and *Cox-2^{luc/-}* progeny. *Cox-2^{luc/+}* and *Cox-2^{luc/-}* macrophages were treated with LPS and assayed for luciferase activity (Fig. 1A). Luciferase activity is induced at two hours after LPS addition and reaches a maximum at four hours in *Cox-2^{luc/+}* macrophages. *Cox-2^{luc/-}* macrophages also show luciferase induction at two and four hours. However, luciferase activity in *Cox-2^{luc/-}* macrophages is only 30 - 50% of that in *Cox-2^{luc/+}* macrophages, despite the fact that both macrophage populations have one *Cox-2^{luc}* allele.

Cox-2 expression is also induced by zymosan in peritoneal macrophages [24]. *Cox-2^{luc/+}* and *Cox-2^{luc/-}* macrophages were treated with zymosan (Fig. 1B). Luciferase expression is induced in macrophages of both genotypes. However, once again, luciferase activity of *Cox-2^{luc/-}* macrophages is only about 50% of that in *Cox-2^{luc/+}* macrophages.

The activating effect of COX-2 protein expression on subsequent *Cox-2* promoter activity in peritoneal macrophages was also observed in an experiment using the COX-2 specific inhibitor, NS398 (Fig. 1C). *Cox-2^{luc/+}* macrophage luciferase activity is decreased to 50% and 30% with 10 nM and 100 nM NS398, respectively. These genetic (Figs 1A, 1B) and pharmacologic (Fig. 1C) results suggest that downstream COX-2 products have a positive feedback effect on *Cox-2* transcription in peritoneal macrophages.

The major downstream COX-2 macrophage product is PGE₂. We examined whether exogenous PGE₂ would restore transcriptional activity of the *Cox-2* promoter in *Cox-2^{luc/-}* macrophages (Fig. 2A). Although, once again, endotoxin-treated *Cox-2^{luc/-}* macrophage expressed only half the *Cox-2* promoter-directed luciferase activity observed in *Cox-2^{luc/+}* macrophages, luciferase activity in *Cox-2^{luc/-}* macrophages is comparable with activity in *Cox-2^{luc/+}* macrophages when the cells receive PGE₂ in addition to LPS. PGE₂ also increased luciferase activity of LPS-treated *Cox-2^{luc/+}* macrophages; greater induction is observed with both 5 nM and 50 nM PGE₂ in macrophages of both genotypes. In contrast, PGD₂ (5 nM or 50 nM) does not rescue *Cox-2* promoter activity in *Cox-2^{luc/-}* macrophages or increase luciferase activity in *Cox-2^{luc/+}* macrophages.

To compare the effect of PGE₂ alone and the combination of PGE₂ plus LPS on *Cox-2* promoter activity, *Cox-2^{luc/-}* macrophages, (which cannot produce any COX-2) were examined for luciferase activity following treatment with each ligand alone and in combination (Fig. 2B). Luciferase activity is induced approximately three-fold by PGE₂ alone, while LPS alone induced a 20 fold increase in luciferase activity. When LPS was added together with PGE₂ the luciferase induction was 40 fold, indicating that PGE₂ and LPS co-operate to elevate *Cox-2* transcriptional activity in murine macrophages.

A number of studies suggest that feedback regulation of *Cox-2* transcription by prostanoid products is varied and dependent on cell type. We previously reported that the absence of *Cox-2* protein expression does not modulate transcriptional activation for the *Cox-2* gene in MEFs; no difference in luciferase activity was observed between *Cox-2^{luc/+}* and *Cox-2^{luc/-}* MEFs induced with TPA [21]. In Figure 3A, we demonstrate this result again, to emphasize the difference in this regard between macrophages (Figs. 1A and 1B) and fibroblasts. To examine the direct effect of PGE₂ on *Cox-2* promoter activity in fibroblasts unable to express any COX-2 enzyme, *Cox-2^{luc/-}* MEFs were treated with TPA and PGE₂ (Fig. 3B). TPA alone induces luciferase activity from the *Cox-2* promoter approximately nine fold. PGE₂ alone does not induce luciferase activity. In contrast to the results for macrophage treated with the combination of LPS and PGE₂, where additional stimulation of LPS-induced *Cox-2* promoter activity was observed in the presence of PGE₂ (Fig. 2), PGE₂ suppresses the TPA-induced luciferase activity elicited from the *Cox-2* promoter in MEFs (Fig. 3B). LPS alone, or the combination of LPS plus PGE₂, does not induce luciferase activity from the *Cox-2* promoter in MEFs, unlike results for macrophage.

Our results with cells isolated from *Cox-2^{luc/+}* and *Cox-2^{luc/-}* mice support the conclusions, established in many cell culture models, that *Cox-2* promoter activity can be modulated by prostanoid products and that the nature of this modulation is cell-type dependent. The use of *Cox-2^{luc/+}* and *Cox-2^{luc/-}* mice, in which luciferase reporter gene expression permits us to monitor non-invasively the *Cox-2* gene transcriptional activity [21], provides a hitherto unavailable opportunity to monitor prostanoid-dependent modulation of *Cox-2* promoter activity/gene expression in living animals. To investigate this feedback system in living mice, we used *in vivo* imaging to compare *Cox-2* promoter-dependent luciferase activity in *Cox-2^{luc/+}* and *Cox-2^{luc/-}* mice following an inflammatory stimulus.

Previously, we optimized a zymosan-induced paw inflammation model for mice, and characterized luciferase reporter responsiveness in *Cox-2^{luc/+}* mice during this inflammation process [21]. To extend this system to the study of prostanoid feedback on *Cox-2* gene expression in living mice, *Cox-2^{luc/+}* mice (which produce COX-2 dependent prostanoids in response to this inflammatory stimulus) and *Cox-2^{luc/-}* mice (which cannot produce COX-2 dependent prostanoids) were injected with zymosan into the right rear paws and with saline into the left rear paws. Luciferase expression was analyzed by non-invasive bioluminescent imaging [21] before zymosan injection and at 6, 9, 12, 24 and 48 hrs following zymosan (Fig. 4A). In *Cox-2^{luc/+}* heterozygous mice, elevated *Cox-2* promoter-driven luciferase signal was observed at six hours in the zymosan-injected paw, reached a maximum level around 9–12 hours, and decreased to baseline levels by 24 hours (Fig. 4, and data from [21]). In *Cox-2^{luc/-}* mice, luciferase activity is also induced at nine hours following zymosan injection and starts decreasing at 12 hours (Fig. 4). However, at 24 hours, the luciferase signal increases again and shows a second, higher peak of luciferase activity not seen in *Cox-2^{luc/+}* mice. The second wave of *Cox-2* transcriptional activity in *Cox-2^{luc/-}* mice decreases by 48 hours.

Discussion

In this study we demonstrated the effect of COX-2 products on *Cox-2* promoter activity, using a *Cox-2* luciferase knock-in/knock-out allele. Luciferase reporter gene expression is regulated by the endogenous chromosomal *Cox-2* promoter region for the *Cox-2^{luc}* knock-in allele, in same manner as the wild-type *Cox-2* gene. Promoter-mediated transcriptional activation of the *Cox-2^{luc}* knockout and *Cox-2⁺* wild-type alleles should be identical, in cultured cells and *in vivo*. The induced luciferase activity of *Cox-2^{luc/luc}* homozygous mouse embryo fibroblasts is approximately double the amount of luciferase activity found in *Cox-2^{luc/+}* fibroblasts, suggesting a simple gene dosage relationship for expression from the *Cox-2^{luc}* allele in those cells [21]. However, *Cox-2^{luc/-}* cells, which are heterozygous for luciferase expression but are homozygous *Cox-2* knockouts, have one *Cox-2^{luc}* allele—like *Cox-2^{luc/+}* cells. As a result, one can study the transcriptional activation of the *Cox-2^{luc}* allele in the presence or absence of a wild-type, functional *Cox-2⁺* allele.

The *Cox-2* gene contains a number of “AREs” (AU-rich elements) in the 3′-untranslated regions (UTR) [25,26]. AREs regulate the stability and translation of many mRNAs, including *Cox-2* mRNA [27]. For example, in IL-1β treated human synovial fibroblasts, PGE₂ regulates *Cox-2* stability post-transcriptionally through the effect of p38 MAP kinase on the 3′-UTR [13]. However, the *Cox-2^{luc}* allele was constructed with an SV40 polyA signal at its 3′ region [21] instead of the original *Cox-2* 3′ non-coding sequence, which contains AREs. Thus, by using the *Cox-2^{luc}* allele, one can monitor the transcriptional activity of the endogenous *Cox-2* gene promoter, eliminating the *Cox-2* gene 3′-UTR effects on message stability and translation.

In peritoneal macrophage, *Cox-2^{luc/-}* cells show less *Cox-2* promoter activity after LPS induction than do *Cox-2^{luc/+}* cells. This difference is rescued by the addition of PGE₂, but not

by PGD₂. PGE₂ induces only a slight increase in *Cox-2* transcription by itself, however, in contrast to its significantly enhancing effect on the transcriptional activation induced by LPS. These results indicate the existence of positive feedback regulation by PGE₂ on *Cox-2* promoter activity in murine macrophages. Mouse peritoneal macrophages express PGE receptor subtypes EP2 and EP4. PGE₂ binding to these receptors increases intracellular cAMP levels, in both untreated and LPS treated mouse peritoneal macrophages [28]. Similarly, in murine bone marrow derived macrophages, *Cox-2* expression is up-regulated by PGE₂ or by dibutyryl cAMP (a cell permeable analogue of cAMP) in association with TNF α [29]. In RAW 264.7 macrophages, PGE₂ up-regulates *Cox-2* expression with LPS; this up-regulation is mediated by adenylyl cyclase [14]. These studies suggest that the positive feedback effect of PGE₂ on *Cox-2* expression in cultured macrophages is mediated by cAMP. Elevated cAMP activates protein kinase A, which phosphorylates CREB, in bone marrow derived macrophages [30]. Although CREB elevation in response to PGE₂ is not by itself able to activate *Cox-2* gene expression, the data suggest that phosphorylated CREB contributes to the enhancement of LPS- or TNF α - induced *Cox-2* gene transcription. Positive feedback regulation of PGE₂ has also been reported in human monocytes, lung fibroblast cells, human cholangiocarcinoma cells, prostate cancer cell lines and mouse cultured podocytes [14-16,31,32].

Transcriptional regulation of the *Cox-2* gene is very complex; substantial feedback variation appears to exist in different cell types and in response to distinct stimuli in individual cell types. Moreover, multiple signaling pathways appear to be interacting with one another. In contrast to PGE₂ effects on COX-2 induction in macrophages, we observe no difference in luciferase induction between *Cox-2^{luc/-}* and *Cox-2^{luc/+}* embryonic fibroblasts treated with TPA. However, exogenous PGE₂ suppresses the TPA-induced *Cox-2* transcription with TPA, suggesting a potential PGE₂ negative feedback regulation in these cells. PGE₂ also down-regulates *Cox-2* expression in human umbilical vein endothelial cell (HUVEC) [33].

Cox-2 expression is also regulated by other prostanoids. PGF_{2 α} , 15d-PGJ₂ and 6-keto PGF_{1 α} enhance COX-2 protein in mouse lung fibroblast cells [15], however, 15d-PGJ₂ negatively regulates COX-2 in the macrophage-like differentiated cell line (U937) and rheumatoid synovial fibroblasts [17,18]. These studies, and our results, indicate that feedback regulation in cultured cells is dependent on cell type.

Because *Cox-2* expression can be induced in many types of cells, and alternative prostanoids have been shown to regulate *Cox-2* expression in cell culture systems, it is quite likely that *Cox-2* expression *in vivo* is also regulated by positive and negative feedback mechanisms in response to prostaglandins produced by various types of cells. Using *Cox-2^{luc/-}* and *Cox-2^{luc/+}* mice makes it possible to analyze, in living animals, COX-2 product-dependent feedback regulation of *Cox-2* gene expression. To investigate whether the transcriptional effects on the *Cox-2* gene by COX-2 dependent products could be monitored in living animals by utilizing *Cox-2^{luc}* mice, we examined luciferase expression during paw inflammation induced by zymosan injection, a model previously described and investigated in our laboratory [21], in *Cox-2^{luc/+}* and *Cox-2^{luc/-}* mice. In this inflammation model, *Cox-2^{luc/+}* mice exhibit a single peak of *Cox-2* promoter driven luciferase activity following zymosan injection. In contrast, *Cox-2^{luc/-}* mice have a second peak of luciferase activity. This second peak of luciferase activity in the zymosan-injected, inflamed paw is not observed in *Cox-2^{luc/+}* mice. This result suggests the existence of negative feedback regulation of *Cox-2* transcriptional activity by one of the COX-2 downstream products; COX-2 dependent prostanoid production appears to prevent the second peak of *Cox-2* promoter activity in the zymosan-injected paw.

In our homogeneous macrophage and fibroblast cell culture systems, the effects of COX-2 dependent prostanoid synthesis on subsequent *Cox-2* transcriptional activity are clearly occurring in the same cell type. However, in the *in vivo* paw inflammation model, it is quite

possible – and even likely – that COX-2 dependent prostanoid production in one cell type will modulate *Cox-2* transcriptional activity in a second cell type. Thus the second peak of *Cox-2* promoter transcriptional activity observed in the paw following zymosan injection may, in fact, occur in a population of cells different from the cells in which the initial *Cox-2* transcriptional activity is seen. We previously demonstrated the local accumulation of COX-2 protein following zymosan injection occurs in epidermal cells and infiltrating inflammatory cells of the zymosan injected paw, using anti-COX-2 immunohistochemistry [22]. We have not yet identified the cell type(s) expressing the initial and subsequent peaks of luciferase activity in *Cox-2^{luc/-}* mice; available anti-luciferase antibodies do not perform well in immunohistochemical studies. Using a different reporter gene such as GFP (e.g. in *Cox-2^{gfp/+}* and *Cox-2^{gfp/-}* mice) or cell-type specific conditional *Cox-2* knockout mice [34] should make it possible to overcome this problem and identify the cells in which *Cox-2* expression is regulated by this feedback mechanism *in vivo*.

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Figure 1.

(A) Peritoneal macrophages were isolated from two *Cox-2^{luc/+}* and two *Cox-2^{luc/-}* mice, treated with LPS (100 ng/ml) for times shown and assayed for luciferase activity. (B) Zymosan-treated macrophage from *Cox-2^{luc/+}* and *Cox-2^{luc/-}* mice were assayed for luciferase activity. Data are averages $\pm$ SD for three independent cultures for each mouse at each time point in (A) and (B). (C) NS398 suppresses *Cox-2^{luc/+}* peritoneal macrophage luciferase activity. Data are averages $\pm$ SD for three independent cultures.

Figure 2.

(A) PGE₂ enhances *Cox-2* driven luciferase expression in *Cox-2*^{*luc*^{+/+} and *Cox-2*^{*luc*^{-/-} macrophages. Macrophages were treated with LPS (100 ng/ml), LPS + PGE₂ (5 or 50 nM) or LPS + PGD₂ (5 or 10 nM) for 4 hours. (B) PGE₂ plus LPS induce an increased transcriptional response. *Cox-2*^{*luc*^{-/-} macrophages were treated with LPS (100 ng/ml), PGE₂ (5 nM) or LPS + PGE₂ for 4 hours. Data are averages ± SD for three independent cultures for (A) and (B).}}}

Figure 3.

(A) Luciferase induction from the *Cox-2* promoter in *Cox-2*^{*luc*^{+/+} and *Cox-2*^{*luc*^{-/-} MEFs treated with TPA (50 ng/ml). (B) Luciferase induction from the *Cox-2* promoter in MEFs by TPA is inhibited by exogenous PGE₂. *Cox-2*^{*luc*^{-/-} MEFs isolated from two different mice were treated with TPA (50 ng/ml), PGE₂ (50 nM), TPA+ PGE₂, LPS (100 ng/ml), and LPS+ PGE₂ for 4 hours. Data are averages ± SD for three independent cultures for each time point for (A) and B).}}}

Figure 4.

(A) *In vivo* imaging of *Cox-2^{luc}^{+/+}* and *Cox-2^{luc}^{-/-}* mice. Each mouse was injected with zymosan in the right rear paw and PBS in the left paw, then anesthetized, injected with luciferin, and imaged at time shown following zymosan injection. (B) Luciferase activities, as photon number, are shown for zymosan-injected paws of each mouse.