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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 PGE_2, induction was comparable, suggesting positive *Cox-2* feedback regulation by PGE_2 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.

The cyclooxygenases (COXs) catalyze the conversion of arachidonic acid to PGH_2, 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), 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 up-regulated in a tissue-specific fashion in *Cox-1* knock-out 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* knock-out 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 knock-out alleles were described previously [21]. LPS (lipopolysaccharide; *Escherichia coli* serotype 0111:B4), zymosan, and TPA (tetradecanoylphorbol acetate) were from Sigma (MO, USA). PGE_2, PGD_2, and NS398 were from Cayman Chemicals (Ann Arbor, MI, USA).

Macrophages were isolated by peritoneal lavage three days after peritoneal injection (3 ml) of 3% thioglycolate medium

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(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, PGE_2, and PGD_2-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, affects 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* knock-out 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 knock-out mice completely lack endotoxin-induced PGE_2 synthesis [23]. *Cox-2*$^{luc/-}$ mice are homozygous for their inability to express functional COX-2 and should, therefore, similarly not produce PGE_2. *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 2 h after LPS addition and reaches a maximum at 4 h in *Cox-2*$^{luc/+}$ macrophages. *Cox-2*$^{luc/-}$ macrophages also show luciferase induction at 2 and 4 h. 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 and 100 nM NS398, respectively. These genetic (Fig. 1A and B) 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 PGE_2. We examined whether exogenous PGE_2 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 PGE_2 in addition to LPS. PGE_2 also increased luciferase activity of LPS-treated *Cox-2*$^{luc/+}$ macrophages; greater induction is observed with both 5 nM and 50 nM PGE_2 in macrophages of both genotypes. In contrast, PGD_2 (5 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 PGE_2 alone and the combination of PGE_2 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 3-fold by PGE_2 alone, while LPS alone induced a 20-fold increase in luciferase activity. When LPS was added together with PGE_2 the luciferase induction was 40-fold, indicating that PGE_2 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 Fig. 3A, we demonstrate this result again, to emphasize the difference in this regard between macrophages (Fig. 1A and B) and fibroblasts. To examine the direct effect of PGE_2 on *Cox-2* promoter activity in fibroblasts unable to express any COX-2 enzyme, *Cox-2*$^{luc/-}$ MEFs were treated with TPA and PGE_2 (Fig. 3B). TPA alone induces luciferase activity from the *Cox-2* promoter approximately 9-fold. PGE_2 alone does not induce luciferase activity. In contrast to the results for macrophage treated with the combination of LPS and PGE_2, where additional stimulation of LPS-induced *Cox-2* promoter activity was observed in the presence of PGE_2 (Fig. 2), PGE_2 *suppresses* the TPA-induced luciferase activity elicited from the *Cox-2* promoter in MEFs (Fig. 3B). LPS alone, or the combination of LPS plus PGE_2, does not induce luciferase activity from the *Cox-2* promoter in MEFs, unlike results for macrophage.

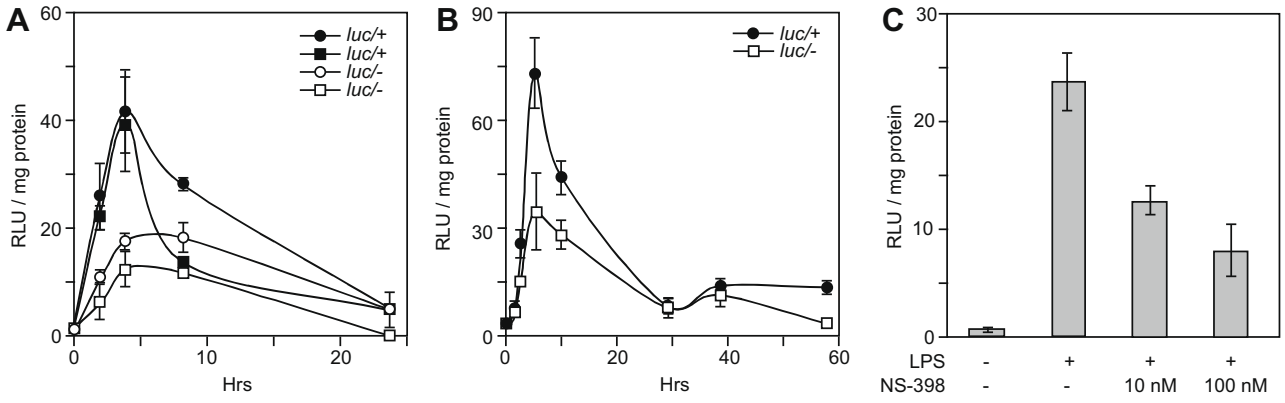

Fig. 1. (A) Peritoneal macrophages were isolated from two *Cox-2*$^{luc/+}$ and two *Cox-2*$^{luc/-}$ mice, treated with LPS (100 ng/ml) for times shown and assayed for luciferase activity. (B) Zymosan-treated macrophage from *Cox-2*$^{luc/+}$ and *Cox-2*$^{luc/-}$ mice were assayed for luciferase activity. Data are averages ± SD for three independent cultures for each mouse at each time point in (A,B). (C) NS398 suppresses *Cox-2*$^{luc/+}$ peritoneal macrophage luciferase activity. Data are averages ± SD for three independent cultures.