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## Cutting Edge: Yin-Yang: Balancing Act of Prostaglandins with Opposing Functions to Regulate Inflammation

Asim K. Mandal,<sup>1</sup> Zhongjian Zhang, Sung-Jo Kim, Pei-Chih Tsai, and Anil B. Mukherjee<sup>2</sup>

For many years, cyclooxygenase-2 (COX-2), a critical enzyme for PG production, has been the favorite target for anti-inflammatory drug development. However, recent revelations regarding the adverse effects of selective COX-2 inhibitors have stimulated intense debate. Interestingly, in the early phase of inflammation, COX-2 facilitates inflammatory PG production while in the late phase it has anti-inflammatory effects. Moreover, although some PGs are proinflammatory, others have anti-inflammatory effects. Thus, it is likely that PGs with opposing effects maintain homeostasis, although the molecular mechanism(s) remains unclear. We report here that an inflammatory PG, PGD<sub>2</sub>, via its receptor, mediates the activation of NF-κB stimulating COX-2 gene expression. Most interestingly, an anti-inflammatory PG (PGA<sub>1</sub>) suppresses NF-κB activation and inhibits COX-2 gene expression. We propose that while pro- and anti-inflammatory PGs counteract each other to maintain homeostasis, selective COX-2 inhibitors may disrupt this balance, thereby resulting in reported adverse effects. *The Journal of Immunology*, 2005, 175: 6271–6273.

Prostaglandins, commonly known as lipid mediators of inflammation, are produced from arachidonic acid by cyclooxygenase (COX)<sup>3</sup> enzymes. There are two isoforms of COX: COX-1 and COX-2. Although COX-1 facilitates the generation of PGs that are essential for maintaining physiological processes, COX-2 generates PGs that are linked to inflammation and cancer (1). Thus, for decades, inhibition of COX-2 has been the target of anti-inflammatory drug development (2). However, several adverse effects of selective COX-2 inhibitors (3) have become the subject of intense debate (4), although the molecular mechanism(s) that mediates these adverse effects remains unclear. Recent reports indicate that during the early phase of agonist-induced inflammation, COX-2 generates primarily inflammatory PGs such as PGD<sub>2</sub> and PGF<sub>2α</sub>, while in the late phase, it may facilitate the produc-

tion of anti-inflammatory PGs (5). It has been reported that cyclopentenone PGs, such as PGA<sub>1</sub>, play important roles in the resolution of inflammation (6). Although it is established that the biological effects of PGs, generated by inflammatory or allergic stimuli, are mediated via heterotrimeric G protein-coupled receptors, it is not clear 1) whether the effects of anti-inflammatory PGs counteract those of inflammatory PGs, and 2) if so, how might the anti-inflammatory PGs exert such effects. The answers to these questions may explain at least some of the adverse effects of selective COX-2 inhibitors (3). In the present study, we report that PGD<sub>2</sub> and PGF<sub>2α</sub> mediate NF-κB activation stimulating the expression of COX-2, which is critical for the generation of proinflammatory PGs. PGA<sub>1</sub>, in contrast, suppresses NF-κB activation and inhibits COX-2 gene expression. Thus, the inflammatory and anti-inflammatory PGs appear to play a yin-yang role to maintain homeostasis.

### Materials and Methods

#### Cell culture and treatment

The NIH 3T3 cells (American Type Culture Collection) were grown in DMEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C with 5% CO<sub>2</sub>. Before the treatment with various effectors (5 μM PGD<sub>2</sub> and 5 μM PGF<sub>2α</sub> or PGA<sub>1</sub>), cells were grown in their respective recommended growth medium to 70–80% confluence, washed once with OptiMem-1 medium containing 2.5% FBS, and then treated with the indicated effectors in OptiMem-1 medium containing 2.5% FBS for 2 h at 37°C with 5% CO<sub>2</sub>.

#### RNA isolation and Northern blot analyses

Total RNAs were isolated using either RNazol B (Tel-Test) following the suppliers' protocol. Thirty micrograms of the total RNA, loaded in each lane, was resolved by electrophoresis on 1.5% formaldehyde-agarose gels. Following electrophoresis, RNAs were transferred to Hybond N<sup>+</sup> (Amersham Biosciences), cross-linked, and hybridized with [ $\alpha$ -<sup>32</sup>P]deoxycytidine triphosphate-labeled cDNA probes at 68°C. Normalization of the amount of RNA loaded was achieved by hybridizing the same blots with GAPDH probe. Mouse and human cDNA probes, used to hybridize the Northern blots, were generated by RT-PCR.

#### Western blot analyses

For detection of proteins, cell lysates were prepared in presence of protease and protein phosphatase inhibitors. Forty micrograms of total protein from each sample was resolved by electrophoresis using 7.5% SDS-polyacrylamide gels

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<sup>3</sup> Abbreviation used in this paper: COX, cyclooxygenase.

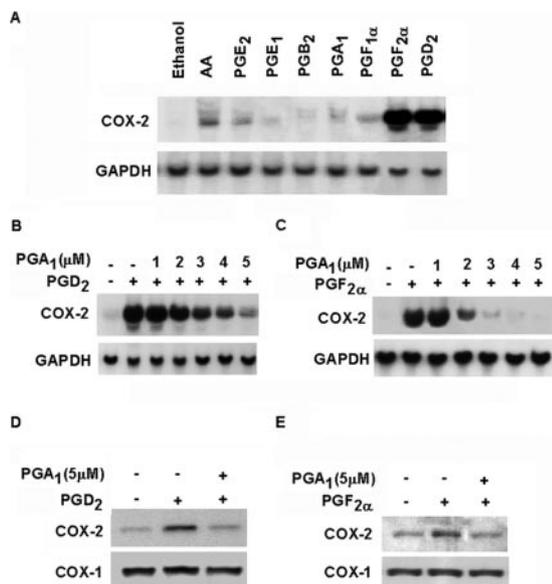
under reducing conditions. Proteins were then electrotransferred to polyvinylidene fluoride membrane (Immobilon P; Millipore). Immunoblot analysis was performed using rabbit polyclonal anti-COX-1/COX-2 (Santa Cruz Biotechnology). HRP-conjugated anti-rabbit IgG was used as the secondary Ab. Chemiluminescent detection was performed by using ECL system (Amersham Biosciences) according to the manufacturer's instructions.

#### Nuclear extract preparation, EMSA, and NF- $\kappa$ B assay

Cells were treated with 5  $\mu$ M PGD<sub>2</sub> or 5  $\mu$ M PGF<sub>2 $\alpha$</sub>  for 1 h in the presence and in the absence of 5  $\mu$ M PGA<sub>1</sub>. Nuclear extracts were prepared using commercially available buffers (GENEKA Biotech) following the protocol provided by the supplier. EMSAs were performed using the nuclear extracts (20  $\mu$ g protein) on a nondenaturing 5% polyacrylamide gel with the following oligonucleotides: mNF- $\kappa$ Bwt: 5'-GAG GGT GAG GGG ATT CCC TTA GTT AGG AC-3'. NF- $\kappa$ B double-stranded oligonucleotides were generated by annealing sense and antisense oligonucleotides. Specificity of protein-DNA complexes was verified by competing with cold NF- $\kappa$ B oligos or by immunoreactivity with polyclonal Abs specific for p65/p50 subunits of NF- $\kappa$ B. The supernatants were tested for the activity of NF- $\kappa$ B using TransAM Flexi NF- $\kappa$ B assay kit (Active motif), according to the manufacturer's recommendations. Ten micrograms of nuclear protein was tested in each well.

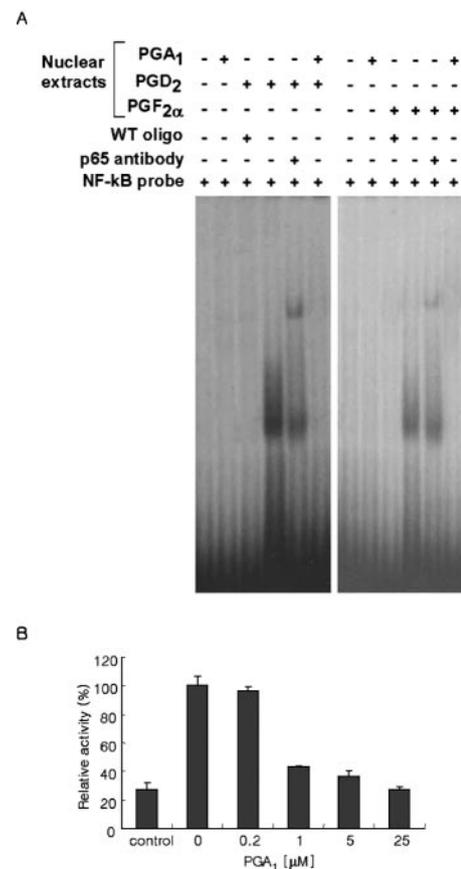
## Results and Discussion

To determine the effects of the pro- and anti-inflammatory PGs on gene expression, we treated NIH 3T3 cells with various PGs and analyzed the level of COX-2 mRNA expression by Northern blot analysis. The results show that while PGD<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  stimulate COX-2 mRNA expression at a high level, other PGs, including cyclopentenone PG, PGA<sub>1</sub>, and arachidonic acid, do not (Fig. 1A). To determine whether the anti-inflammatory cyclopentenone PG, PGA<sub>1</sub>, counteracts the effects of PGD<sub>2</sub> and PGF<sub>2 $\alpha$</sub> , we treated the cells with these PGs in the presence and in the absence of PGA<sub>1</sub>. The results show that while in the absence of PGA<sub>1</sub> both PGD<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  stimulate the expression of COX-2 mRNA (Fig. 1, B and C) and COX-2 protein (Fig. 1, D and E), the presence of PGA<sub>1</sub> suppresses the expression of COX-2 mRNA and protein expression.



**FIGURE 1.** PGA<sub>1</sub> inhibits PGD<sub>2</sub>- and PGF<sub>2 $\alpha$</sub> -mediated COX-2 expression. Northern blot analysis demonstrates PGD<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  treatment (for 2 h at 37°C) stimulate COX-2 mRNA expression in NIH 3T3 cells (A). PGA<sub>1</sub> inhibits the PGD<sub>2</sub>- and PGF<sub>2 $\alpha$</sub> -induced COX-2 mRNA expression in a dose-dependent manner (B and C). Western blot analysis demonstrates that PGA<sub>1</sub> inhibits PGD<sub>2</sub>- and PGF<sub>2 $\alpha$</sub> -induced COX-2 protein expression (D and E) cells.

It has been reported that PGD<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  exert their biological effects via G protein-coupled receptors, DP and FP, respectively (reviewed in Ref. 7). Because activation of NF- $\kappa$ B by PGD<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  via these receptors stimulates COX-2 gene expression (Ref. 8; A. K. Mandal et al., manuscript in preparation), we performed EMSA to determine whether PGA<sub>1</sub> treatment of the cells inhibits PGD<sub>2</sub>- and/or PGF<sub>2 $\alpha$</sub> -mediated NF- $\kappa$ B activation. The results show that in both cell lines PGA<sub>1</sub> suppresses NF- $\kappa$ B activation (Fig. 2A). We further performed a NF- $\kappa$ B activity assay, and our results demonstrate that PGA<sub>1</sub> suppresses NF- $\kappa$ B activity in the cells (Fig. 2B). Taken together, these results strongly suggest that inflammatory PGs via receptor-mediated pathways mediate the activation of NF- $\kappa$ B, thereby stimulating the expression of COX-2, a critical enzyme for the production of proinflammatory lipid mediators. In this scenario, the anti-inflammatory PGA<sub>1</sub> counteracts these effects by suppressing the activation of NF- $\kappa$ B, which is essential for COX-2 gene expression. The physiological roles of cyclopentenone PGs such as PGA<sub>1</sub> are not clearly established as the levels of these PGs are somewhat difficult to determine because conversion of PGE<sub>2</sub>, PGE<sub>1</sub>, and PGD<sub>2</sub> to PGA<sub>1</sub> may occur in vitro by dehydration within the cycloheptane ring (9). Undoubtedly, further investigations establishing the levels of PGA<sub>1</sub> by mass



**FIGURE 2.** PGA<sub>1</sub> inhibits DP- and FP-mediated NF- $\kappa$ B activation. An EMSA using nuclear extracts from control and PGD<sub>2</sub>- or PGF<sub>2 $\alpha$</sub> -treated (for 1 h at 37°C) cells and <sup>32</sup>P-labeled double-stranded NF- $\kappa$ B in the absence and in the presence of respective double-stranded competitor unlabeled oligo, demonstrating the effect of DP- and FP-signaling on the activation of NF- $\kappa$ B. This activation of NF- $\kappa$ B by PGD<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  is inhibited in presence of PGA<sub>1</sub> (A). PGA<sub>1</sub> inhibits the PGD<sub>2</sub>-induced NF- $\kappa$ B activity in the cells. The control bar represents the non-PGD<sub>2</sub>-induced NF- $\kappa$ B activity (B).

spectrometry may allow quantitation of the physiological levels of this PG and its role in suppressing inflammation. Nevertheless, our results at least in part provide a proof of principle that  $\text{PGA}_1$  inhibits COX-2 gene expression via suppression of NF- $\kappa$ B activation. We propose that a balance between the levels of pro- and anti-inflammatory PGs maintains homeostasis and disruption of this balance, which may occur due to preferential inhibition of COX-2 activity by selective COX-2 inhibitors, may underlie the adverse effects of these agents.

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## Disclosures

The authors have no financial conflict of interest.

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