Compensatory Prostaglandin \(\text{E}_2\) Biosynthesis in Cyclooxygenase 1 or 2 Null Cells

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Summary

Prostaglandin \(\text{E}_2\) (PGE\(_2\)) production in immortalized, nontransformed cells derived from wild-type, cyclooxygenase 1-deficient (COX-1\(^{-/-}\)) or cyclooxygenase 2-deficient (COX-2\(^{-/-}\)) mice was examined after treatment with interleukin (IL)-1\(\beta\), tumor necrosis factor \(\alpha\), acidic fibroblast growth factor, and phorbol ester (phorbol myristate acetate). Compared with their wild-type counterparts, COX-1\(^{-/-}\) or COX-2\(^{-/-}\) cells exhibited substantially enhanced expression of the remaining functional COX gene. Furthermore, both basal and IL-1-induced expression of cytosolic phospholipase A\(_2\) (cPLA\(_2\)), a key enzyme regulating substrate mobilization for PGE\(_2\) biosynthesis, was also more pronounced in both COX-1\(^{-/-}\) and COX-2\(^{-/-}\) cells. Thus, COX-1\(^{-/-}\) and COX-2\(^{-/-}\) cells have the ability to coordinate the upregulation of the alternate COX isozyme as well as cPLA\(_2\) genes to overcome defects in prostaglandin biosynthetic machinery. The potential for cells to alter and thereby compensate for defects in the expression of specific genes such as COX has significant clinical implications given the central role of COX in a variety of disease processes and the widespread use of COX inhibitors as therapeutic agents.

Prostaglandins, such as prostaglandin (PG)E\(_2\), are pivotal modulators of tissue homeostasis and their aberrant regulation is known to cause a variety of pathophysiological consequences (1–4). PGE\(_2\) biosynthesis is regulated by successive metabolic steps involving the phospholipase A\(_2\)-mediated release of arachidonic acid (AA) and its conversion to PGE\(_2\) by cyclooxygenase (COX), hydroperoxidase, and isomerase activities (1–4). Although cytosolic phospholipase A\(_2\) (cPLA\(_2\)) is primarily responsible for agonist-induced AA release from membrane phospholipids (5, 6), secretory PLA\(_2\) may also be important in regulating AA availability via a transcellular mechanism (7). Conversion of AA to PGH\(_2\), the committed step in prostanooid biosynthesis, is mediated by cyclooxygenases, COX-1 and -2, which are encoded by two unique genes, located on different chromosomes (2). Generally, although COX-1 is constitutively expressed, the expression of COX-2 is highly inducible (2, 3). Based on their respective modes of expression, it is thought that COX-1 is primarily involved in cellular homeostasis, whereas COX-2 plays a major role in inflammation and mitogenesis. The COX isoenzymes are thought to be the primary target enzymes for nonsteroidal antiinflammatory drugs (NSAIDs), which act by inhibiting the COX activity of COX-1 and -2, thereby blocking their ability to convert AA to PGH\(_2\) (8, 9). In addition to the use of nonsteroidal antiinflammatory drugs as analgesics and for alleviation of acute and chronic inflammation, these agents have proven effective in decreasing the frequency of heart attacks and strokes (8, 10, 11), and in reducing the incidence of colon cancer (12, 13).

Since most cells invariably express both COX-1 and -2 under the appropriate conditions, it has been somewhat problematic to determine the exact contribution of the two COX isozymes towards basal and agonist-inducible PGE\(_2\) biosynthesis. The use of selective COX-1/COX-2 inhibitors to define relative contributions of the two isozymes has also resulted in limited success. The purpose of this study was to examine the effects of COX deficiency on the differential expression of the COX-1 and -2 isozymes, and compare the responses of wild-type, COX-1, and COX-2 knockout cells with respect to agonist-induced PGE\(_2\) bio-

\(^{1}\)Abbreviations used in this paper: AA, arachidonic acid; COX, cyclooxygenase; cPLA, cytosolic phospholipase; FGF, fibroblast growth factor; PG, prostaglandin; RIA, radioimmunoassay.
synthesis. We demonstrate that the expression of COX, cPLA2, and PGE2 production are significantly increased in COX-deficient cells. Thus, COX deficiency, regardless of whether it is COX-1 or -2, results in the enhanced basal and inducible expression of the remaining COX isozyme as well as the elevated expression of cPLA2. We interpret these data to indicate that the elevated production of PGE2 in COX-1 or -2 isozyme-deficient cells is due to the compensatory expression of the remaining COX isozyme.

Materials and Methods

Isolation and Culture of COX-Deficient Mouse Cells. Lungs were collected from wild-type C57BL/6J (B6), COX-1-deficient (14), and COX-2-deficient (15) mice. The tissues were dissected into small pieces and grown underneatheal covergels in 10-cm plates with MEM supplemented with PenStrep at a concentration of 300,000 U/liter penicillin G and 300 mg/liter streptomyacin sulfate, nonessential amino acids (0.1 mM), Fungizone (1 mg/liter Amphotericin B), glutamine (292 mg/liter), acetic acid (50 mg/liter), and 10% FCS in a humidified incubator with 5% CO2 and the media were replaced three times per week. After 3 wk of culture under these conditions, only fibroblasts continued to grow. The PenStrep was reduced to 100,000 U/liter penicillin G and 100 mg/liter streptomyacin sulfate and the media were replaced twice per week for another 3-5 wk. During subsequent passages, cells were maintained in DMEM containing high glucose and COX-deficient cells. Thus, COX deficiency, regardless of whether it is COX-1 or -2, results in the enhanced basal and inducible expression of the remaining COX isozyme as well as the elevated expression of cPLA2. We interpret these data to indicate that the elevated production of PGE2 in COX-1 or -2 isozyme-deficient cells is due to the compensatory expression of the remaining COX isozyme.

Statistical Analysis. Paired t tests were used to determine the differences in the PGE2 levels between control samples of wild-type, COX-1/- or COX-2/- cells, and between control samples and samples from cytokine-treated cells. Differences were considered significant if P < 0.05.

Results and Discussion

We examined the effects of IL-1 on PGE2 production in cells containing both COX isozymes (wild type) compared with cells that had only COX-1 (COX-2/-) or COX-2 (COX-1/-), respectively. As shown in Fig. 1A both COX-1/- or COX-2/- cells synthesized 6-8-fold higher levels of PGE2 compared with their wild-type counterparts. Interestingly, basal PGE2 production was higher in both COX-2/- (66.71 ± 3.54 pg/10^6 cells n=6) and COX-1/- (90.23 ± 3.29 pg/10^6 cells n=8) cells compared with wild-type (11.07 ± 0.62 pg/10^6 cells n=8). All values are mean ± SE.) IL-1 treatment of wild-type and COX-1/- cells further enhanced their PGE2 output. In contrast, IL-1 treatment of COX-2/- cells did not significantly enhance PGE2 biosynthesis (Fig. 1A).

These dramatic differences in basal PGE2 biosynthesis between wild-type and COX-deficient cells prompted us to compare the expression of genes encoding three key en-
Next, we examined the basal and IL-1-stimulated levels of COX-1 protein in identically treated wild-type, COX-2/−/−, and COX-1/−/− cells. Treatment of cells with IL-1/− cells as described in Materials and Methods showed a slight increase in COX-1 protein levels (2.4-fold), but the increase in COX-2 protein was much more dramatic in COX-1/−/− cells (Fig. 1B). The elevated level of COX-2 protein correlates well with the higher basal PGE2 levels in COX-1/−/− cells compared with those in wild-type cells. When treated with IL-1, COX-2 protein levels increased moderately in wild-type cells (Fig. 1B), but the increase in COX-2 protein was much more dramatic in COX-1/−/− cells (41-fold). The overall pattern of COX-2 protein expression in wild-type and COX-1/−/− cells correlated with increased PGE2 production seen in cells with unique COX phenotypes (see Fig. 1A).

Next, we examined the basal and IL-1-stimulated levels of COX-1 protein in identically treated wild-type, COX-2/−/−, and COX-1/−/− cells, respectively (Fig. 1C). In wild-type cell extracts, the level of COX-1 protein was barely detectable and IL-1 treatment was apparently inconsequential. This result was not unexpected since COX-1 expression is not known to be inducible under many conditions. We observed that basal expression of COX-1 protein in untreated COX-2/−/− cells (Fig. 1C) was much greater (14-fold) than that in wild-type cells. This overexpression of COX-1 protein corresponds with greater basal PGE2 levels in COX-2/−/− cells, compared with the basal levels in wild-type cells. IL-1 had no stimulatory effect on COX-1 protein levels in COX-2/−/− cells (Fig. 1C) and as expected, COX-1/−/− cells did not express detectable COX-1 protein.

Another important enzyme in the prostaglandin biosynthetic pathway is PGE2 synthase, the isomerase that converts PGH2 to PGE2. Although PGE2 synthase has neither been sequenced nor cloned, making it difficult to study, available evidence does seem to indicate that this enzyme is not a rate-limiting reaction in PGE2 biosynthesis. However, based upon our findings, we cannot rule out the possibility that PGE2 synthase expression may also be altered in COX null cells.

To examine the possibility that iso-PGE2 or other isoprostanes (18) may be generated nonenzymatically from a buildup of endoperoxide intermediate that cross-reacts with the anti-PGE2 used in our RIA leading to erroneously high estimations of COX and/or PGE2 synthase activity, we performed two experiments. First, we treated wild-type and COX-1/−/− cells with either indomethacin or NS-398, COX-1, and COX-2 selective inhibitors, respectively since these COX inhibitors should block PGE2 synthesis without affecting iso-PGE2 formation. We found that either indomethacin or NS-398 completely blocked both the basal and cytokine-induced formation of immunoreactive PGE2 in wild-type and COX-1/−/− cells (data not shown). Second, radio-thin-layer chromatography was used to confirm that PGE2 was by far the predominant prostanoid product generated by wild-type and COX-1/−/− cells and that no other AA metabolites in addition to PGE2 were generated in COX-1/−/− cells (data not shown).

To compare the effects of IL-1 (see Fig. 1A) to other inducers of PGE2 biosynthesis, we tested the effects of TNF, acidic FGF, and PMA on PGE2 production in wild-type, COX-2/−/−, and COX-1/−/− cells (Fig. 2). Compared to stimulated wild-type cells, there was significantly more PGE2 produced in either COX-1/−/− or COX-2/−/− cells, with the possible exception of TNF that induced comparable PGE2 biosynthesis in each cell type. In response to FGF, the amount of PGE2 that was produced by COX-2/−/− cells was elevated and in COX-1/−/− cells, PGE2 was even more dramatically elevated as compared to wild type. Both COX-2/−/− and COX-1/−/− cells treated with PMA also produced more PGE2 than wild type. Thus, in general, COX-isozyme deficiency results in increased PGE2 biosynthesis, but the relative contributions of COX-1 and COX-2 are clearly dependent upon the specific agonists involved.

Since constitutive COX-2 protein expression and PGE2 production in COX-1/−/− cells was significantly enhanced, we were also curious about the status of cPLA2 gene expression in COX-deficient cells. We reasoned that cPLA2 activity could be involved in regulating levels of free AA for conversion to PGE2 and thereby could play a critical role in compensating for COX-isozyme deficiency. We were
somewhat surprised to find that basal levels of cPLA2 protein in either COX-2/−/− (Fig. 3 B) or COX-1/−/− (Fig. 3 C) cells were significantly higher than levels of cPLA2 in wild-type cells (Fig. 3 A). It is conceivable, therefore, that enhanced expression of cPLA2 could directly contribute to higher PGE2 levels in both of the COX-deficient cells by generating greater AA substrate for PGE2 biosynthesis. Treatment of COX-1/−/− or COX-2/−/− cells with IL-1 resulted in a modest increase in the amount of cPLA2 protein (4-fold in COX-1/−/− and 1.4-fold in COX-2/−/−). This was in contrast to wild-type cells, which showed no change in the levels of cPLA2 protein after treatment with IL-1. As an important control, we examined the quantitative parameters of PGE2 production, and COX-1, COX-2, and cPLA2 gene expression in wild-type, COX-2/−/−, and COX-1/−/− cells from primary cell cultures and found essentially the same patterns in primary cells as those observed in the immortalized cells (data not shown). Therefore, the characteristic pattern of expression of COX-1, COX-2, and cPLA2 proteins in COX-2/−/− and COX-1/−/− cells is not elicited as a result of immortalization caused by the E1A adenovirus gene. Taken together these data indicate that COX-1/−/− cells express enhanced levels of both basal and cytokine-stimulated COX-2 protein, and increased basal expression of cPLA2 protein. We postulate that the significantly increased levels of COX-2 and cPLA2 in COX-1/−/− cells are likely to account for the increased rates of PGE2 biosynthesis; these data also implicate the existence of compensatory mechanisms for PGE2 production in COX-isozyme-deficient cells.

To distinguish between preferences of COX-1 and -2 for endogenous and/or exogenous AA for conversion to PGE2, and to verify that COX-1 was indeed expressed in COX-2/−/− cells as judged by its ability to synthesize PGE2, we added exogenous AA to wild-type, COX-2/−/−, or COX-1/−/− cells that were either untreated or treated with IL-1, TNF, FGF, or PMA. The results shown in Fig. 4 demonstrate that cells expressing only COX-1 (COX-2/−/−) synthesized similar amounts of PGE2 as wild-type or COX-1/−/− cells supplemented with exogenous AA. Therefore, COX-1 is expressed and enzymatically active in COX-2/−/− cells, but cytokines neither enhance COX-1 protein biosynthesis nor PGE2 biosynthesis. Thus, agonists that induce PGE2 biosynthesis in COX-2/−/− cells in the absence of exogenous AA, do so by increasing endogenous substrate availability. Based on these data, we conclude that

### Figure 2. The effect of cytokines and PMA on PGE2 biosynthesis

Wild-type (A), COX-2/−/− (B), and COX-1/−/− (C) mouse cells were treated with IL-1 (0.25 ng/ml), TNF (5 ng/ml), FGF (10 ng/ml), or PMA (12.5 ng/ml) as described in Materials and Methods. After 24 h of culture, media was collected and analyzed for PGE2 by RIA. Data are means ± SE of at least six separate determinations (wells). * Values that are significantly different from the control values of each respective cell type by paired t test (P < 0.05).

### Figure 3. Expression of cPLA2 in IL-1-stimulated cells

Wild-type, COX-2/−/−, and COX-1/−/− mouse cells were treated with vehicle (Control) or with IL-1 (0.25 ng/ml) as described in Materials and Methods. After 24 h of culture, media was removed and Western blot analysis was carried out as described in Materials and Methods. (A) Proteins from wild-type cells. (B) Proteins from COX-2/−/− cells. (C) Proteins from COX-1/−/− cells. A, B, and C are from the same blot, which is a representative of three replications.
in COX-2−/− cells, substrate is likely to be limiting for constitutively expressed COX-1–mediated PGE2 biosynthesis. Fig. 4 also shows that COX-1−/− cells are able to use both exogenous and endogenous substrates (also see Fig. 1 A). However, IL-1, TNF, and FGF significantly enhanced the ability of COX-1−/− cells to produce PGE2, most likely by enhancing COX-2 expression as shown in Fig. 2. In addition, COX-1−/− cells treated with PMA did not produce elevated levels of PGE2, even when exogenous AA was provided. This indicates that PMA likely increased PGE2 production by increasing the availability of endogenous AA in COX-1−/− cells, whereas IL-1, TNF, and FGF likely affect AA mobilization and COX-2 expression. PMA affected the wild-type cells similarly. These results clearly raise the possibility that in COX-1 or -2 null cells, there is a coordinate upregulation of the expression and/or activities of COX-1, COX-2, and cPLA2, leading to increased PGE2 biosynthesis. These data also demonstrate that both COX-1−/− and COX-2−/− cells can effectively use AAs from either endogenous or exogenous sources.

Our data are consistent with the hypothesis that the long-term of COX-isozyme deficiency results in the altered expression of the remaining two enzymes that regulate mobilization and conversion of arachidonic acid to prostaglandins. Fig. 5 summarizes the patterns of COX and cPLA2 expression in COX null cells compared with normal cells in response to IL-1. The scheme shows the compensatory expression of the alternative COX isozyme and cPLA2 when one of the COX isozymes is absent. In cells lacking the housekeeping isozyme COX-1, overcompensation results in the overexpression of COX-2 and cPLA2, and in turn elevated PG biosynthesis. Similarly, cells lacking the inducible isozyme, COX-2, elicit the enhanced expression of COX-1 and cPLA2. Although we are unable to comment as to the precise status of PGE2 synthase expression in wild-type, COX-1−/− or COX-2−/− cells, we have depicted its expression in each cell type; since PGE2 is the predominant prostanoïd product, its expression would not appear to be rate-limiting given the great potential for PGE2 biosynthesis in the presence of exogenous AAs (see Fig. 4).

Thus, our data clearly show that COX-deficient cells have the potential to overcome the lack of expression of one or the other COX isoenzymes by overexpressing the alternate COX isoform and increased cPLA2 expression. Such a potential mechanism for producing PGE2 by cells in vitro is not surprising since neither COX-1−/− (14) nor COX-2−/− mice (15) showed severe developmental arrest in utero or immediate postnatal mortality. However, in contrast to the results shown here using lung fibroblasts, Langenbach et al. (14) did not report any compensatory COX-2-mediated PGE2 production in glandular stomachs of COX-1-deficient mice, suggesting that tissue specificity may also be an important factor for further investigation. Together, these findings underscore the importance of elucidating the potential long-term effects of COX-1 or COX-2 inhibition with respect to alterations in the quantitative and/or qualitative patterns of AA metabolism.

Figure 4. The effect of exogenous arachidonic acid on cytokines or PMA-induced PGE2 production. Wild-type (A), COX-2−/− (B), and COX-1−/− (C) mouse cells were treated with IL-1 (0.25 ng/ml), TNF (5 ng/ml), FGF (10 ng/ml), or PMA (12.5 ng/ml) as described in Materials and Methods. After 24 h of culture, media was removed, the cells were washed to remove any accumulated PGE2, and fresh serum-free media containing AA (50 μM) was added. After a 15-min incubation in the presence of added AA, media was collected and PGE2 measured by RIA. Data are means ± SE of at least six separate determinations (wells). * Values that are significantly different from the control values of each respective cell type by paired t test (P < 0.05).
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Figure 5. The coordinate regulation of COX-1, COX-2, and cPLA2 expression in COX null cells by IL-1. In wild-type cells, inducible COX-2 is responsible for the increase in IL-1-induced PGE2 production as represented by proportionally larger characters and lines throughout the diagram. Although the constitutive level of COX-1 expression is low, exogenous AA (exo. AA) is effectively converted to PGE2. In COX-2-/- or COX-1-/- cells, the overexpression of cPLA2 may play a role in increased basal PGE2 biosynthesis compared to wild-type cells by increasing the availability of endogenous AA (endo. AA). IL-1 greatly induces COX-2 accumulation in COX-1-/- cells resulting in enhanced PGE2 biosynthesis. In COX-2-/- cells, overexpression of COX-1 and cPLA2 lead to an increase in basal PGE2 biosynthesis compared to wild type. However, IL-1 does not enhance PGE2 biosynthesis in COX-2-/- due to the lack of increased COX-1 expression. As in wild-type cells, exogenous AA is effectively used by COX-1 in COX-2-/- cells as indicated by a high level of PGE2 accumulation. At present, the effects of COX-isozyme deficiency on the expression of PGE2 synthase are not known, but this enzyme does not appear to be rate limiting.

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