Potential risks of ovarian cancer: a systematic overview of the literature. Gori Niccolini, Antonietta Lucchini, Maria Camilla Cipriani, Elena Negri, and Rose Maria Negri

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Molecular Dynamics, and all measurements of COX-2 mRNA were corrected to the internal standard GAPDH and are reported as ratios.

**Western Analysis.** NIH 3T3 cells were treated with PGE$_2$ or PGE$_3$ at various concentrations and for various times as described in the figure legends. After the treatment with PG, the cells were washed once with PBS and lysed in passive lysis buffer (Promega). Fifteen micrograms per sample was electrophoresed on an 8% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. Primary antibodies to COX-2 (Cayman Chemicals) and GAPDH (Chemicon) were used at the manufacturer’s recommended dilutions. Secondary horseradish peroxidase-linked anti-rabbit IgG antibody was used at a dilution of 1:12,000. Bands were visualized with an enhanced chemiluminescence detection reagent (Amersham Pharmacia).

**Cell Proliferation Assay.** The cell growth assay was performed by using the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega). NIH 3T3 cells were seeded in 96-well plates at low density (10,000 cells per well) in DMEM, allowed to attach overnight, and made quiescent by a 24-h incubation in 0.5% serum medium. Cells were then treated with different concentrations of PGE$_2$ or PGE$_3$. After 24 h, Cell Titer 96 Aqueous One Solution reagent was added to each well, and the plate was incubated for 4 h after which the absorbance was recorded at 490 nm with a 96-well plate reader.

**Transient Transfections.** Murine NIH 3T3 cells at 50–60% confluency in 6-well dishes were transfected with 2 µg of total DNA per well by using Superfect (Qiagen) for 2 h. All DNAs were prepared with Endotoxin-Free Plasmid Preparation kits (Qiagen). All transfections also included 0.1 µg per well of plr-TK (a plasmid encoding Renilla luciferase, used as a transfection efficiency control; from Promega). After this procedure, the cells were washed once with PBS and incubated overnight in DMEM supplemented with 0.5% heat-inactivated FCS. After 24 h, the cells were treated for 6 h with 20% FBS, PGE$_2$, and PGE$_3$. After the treatment, cells were washed twice with PBS and lysed with 1× Passive Lysis buffer (Promega).

**Luciferase Assays.** Luciferase activity in cell lysates was measured with the Dual Luciferase kit (Promega). Relative luciferase activity of purified cell extracts was typically represented as (firefly luciferase value/Renilla luciferase value) × 10$^3$.

**Other Methods.** PGE$_2$ concentrations in the conditioned media were determined with a PGE$_2$ EIA kit (Cayman Chemical). IL-6 concentrations in the supernatants were determined by using an ELISA kit (BioSource International). Protein concentrations of cell extracts were measured by using the Bradford reagent (Bio-Rad).

**Results**

**PGE$_2$ Is Not Mitogenic to NIH 3T3 Fibroblasts.** We first compared the effect of PGE$_2$ and PGE$_3$ on cell proliferation. PGE$_2$ at a concentration of 50 ng/ml or greater stimulated proliferation of NIH 3T3 cells. In contrast, PGE$_3$ up to 1 µg/ml did not affect cell proliferation (Fig. 1B).

**PGE$_2$ and PGE$_3$ both Induce COX-2 mRNA in NIH 3T3 Fibroblasts.** PGE$_2$-dependent amplification of COX-2 (10–14) is hypothesized to be an important part of sustained proliferative and chronic inflammatory conditions, and may explain the overexpression of COX-2 in tumors (21). We next examined the effect of PGE$_2$ on COX-2 expression. Interestingly, both PGE$_2$ and PGE$_3$ induce COX-2 message in a time- and dose-dependent fashion (Fig. 2). COX-2 message peaks at 1 h after induction with either PGE$_2$ or PGE$_3$ and is detectable at a concentration as low

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**Figure 1.** (A) Structures of AA (20:4, ω-6), EPA (20:5, ω-3), and their oxygenated COX metabolites PGE$_2$ and PGE$_3$, respectively. (B) PGE$_2$, but not PGE$_3$, stimulates NIH 3T3 cell proliferation. Cells were treated with PGE$_2$ or PGE$_3$ at various concentrations. Cell proliferation was measured after 24 h. Data are the means ± SD from triplicate wells.
as 50 ng/ml for both PGE₂ and PGE₃. However, PGE₃ is less potent compared with PGE₂. At all concentrations tested, PGE₂ induced COX-2 up to 4-fold more than PGE₃. Furthermore, although both PGE₂ and PGE₃ also induce the accumulation of COX-2 protein in a time- and dose-dependent manner (Fig. 3), similar to the mRNA, COX-2 protein levels achieved with PGE₂ treatment were substantially higher compared to those achieved with PGE₃.

Transcriptional Activation of COX-2 by PGE₂ and PGE₃. In transient transfections, using WT COX-2 promoter (-724/+7) linked to a luciferase reporter gene, PGE₂ (1 μg/ml) induced COX-2-dependent luciferase activity by >8-fold (Fig. 4A). Although PGE₃ also induced COX-2-dependent luciferase activity, it was less effective at inducing COX-2 promoter activity compared to PGE₂ (Fig. 4A). Two NF-IL6 sites and a cyclic-AMP response element (CRE) are present between -724 bp and +7 bp of the COX-2 promoter, and two Ras-mediated signal transduction pathways (Ras/JNK and Ras/ERK) are required for the regulation of COX-2 gene expression in platelet-derived growth factor (PDGF)-treated NIH 3T3 cells (22), mast cells activated by the aggregation of IgE receptors (20), and in MC3T3-E1 cells treated with basic fibroblast growth factor or PDGF (23). We next examined the cis-acting elements and the signal transduction pathways necessary for COX-2 induction by PGE₂ and PGE₃ in NIH 3T3 fibroblasts. PGE₂- and PGE₃-dependent luciferase activity was significantly inhibited in COX-2 promoter mutant constructs in which either both NF-IL6 sites were mutated or the CRE site was mutated (Fig. 4B). Mutation of

Fig. 2. Time course and dose–response of induction for COX-2 mRNA by PGE₂ and PGE₃. NIH 3T3 fibroblasts were treated for 1 h with different concentrations of PGE₂ or PGE₃ (A), or with either PGE₂ (1 μg/ml) or PGE₃ (1 μg/ml) for different time periods (B). Total RNA was isolated, and Northern analysis for COX-2 and GAPDH was performed as described in Experimental Procedures. (Right) Quantified data normalized to GAPDH.

Fig. 3. Time course and dose–response of induction for COX-2 protein by PGE₂ and PGE₃. NIH 3T3 fibroblasts were treated for 4 h with different concentrations of PGE₂ or PGE₃ (A), or with either PGE₂ (1 μg/ml) or PGE₃ (1 μg/ml) for different time periods (B). Total protein was harvested, and 15 μg per condition was subjected to Western blotting with anti-COX-2 antibody as described in Experimental Procedures.
E-BOX or the NFκB site (data not shown) did not affect PGE$_2$- or PGE$_3$-dependent luciferase activity. To determine the signal transduction pathways necessary for COX-2 induction by PGE$_2$ or PGE$_3$, NIH 3T3 cells were cotransfected with a WT COX-2 reporter and with either a control plasmid or plasmids expressing DN-Ras, DN-JNK, or DN-ERK1 (C). The transfected cells were treated with serum (20%), PGE$_2$, or PGE$_3$ at the concentrations indicated. Cells were harvested after 6 h and assayed for luciferase activity and total protein. Values are means ± SD. *, $P < 0.05$ vs. PGE$_2$ treatment.

Alteration of $\omega$-6 Composition of Membrane Phospholipids in NIH 3T3 Fibroblasts Enhances COX-2-Dependent PGE$_2$ Synthesis. It has been shown that both $\omega$-3 and $\omega$-6 long-chain PUFA added exogenously to cells in culture become part of the membrane phospholipid pool (24, 25). Accumulation of exogenously added PUFA occurs in the phospholipid component of the membranes during the first 8–16 h of exposure, after which there was little

or no accumulation. Moreover, ligand-induced PG synthesis in murine fibroblasts and macrophages depends on both the endogenous AA released from membrane phospholipid stores as well as the synthesis and activity of the COX-2 protein (26). To determine whether membrane composition of fatty acids can affect ligand-induced substrate availability and COX-2-dependent PG synthesis, we first incubated NIH 3T3 fibroblasts with AA, EPA, or both. After 24 h, the cells were washed and stimulated with phorbol 12-myristate 13-acetate (PMA) for 6 h to determine COX-2-dependent PG synthesis. Basal PG synthesis was not significantly affected by modifying the membrane phospholipid with AA (10 $\mu$M), EPA (10 $\mu$M), or both. PGE$_2$ synthesis was significantly increased after PMA stimulation in AA-treated cells (Fig. 5A). However, no such increase was observed in PMA-stimulated cells treated with EPA (Fig. 5A). To be certain that modification of fatty acid composition of membranes did not affect signals initiated by PMA for induction of COX-2 gene expression, we analyzed COX-2 mRNA levels in NIH 3T3 cells that had been treated with fatty acids. There was no difference in PMA-induced accumulation of COX-2 message after treat-
in PGE2 synthesis between the two cell populations was not due to stimulation with PMA (Fig. 6A). Conditioned medium from NIH 3T3 fibroblasts treated with AA and stimulated with PMA induces IL-6 secretion in RAW 264.7 macrophages. Membrane fatty acid composition of NIH 3T3 cells was modified by incubating the cells with AA (10 μM) or EPA (10 μM) for 24 h. Exogenous fatty acid was removed by washing the cells with PBS. Cells were stimulated with or without PMA (50 ng/ml) for 6 h, and the conditioned medium was used to treat the RAW 264.7 macrophages for 24 h. IL-6 secretion by the macrophages was measured by ELISA. Each value represents the mean ± SD.

### Fig. 6. (A) Effect of PGE2 and PGE3 on IL-6 secretion in RAW 264.7 macrophages. Macrophages were treated with either PGE2 (50 ng/ml) or PGE3 (50 ng/ml). IL-6 in the supernatants was determined by ELISA at the indicated time points. (B) Conditioned medium from NIH 3T3 fibroblasts treated with AA and stimulated with PMA induces IL-6 secretion in RAW 264.7 macrophages. Membrane fatty acid composition of NIH 3T3 cells was modified by incubating the cells with AA (10 μM) or EPA (10 μM) for 24 h. Exogenous fatty acid was removed by washing the cells with PBS. Cells were stimulated with or without PMA (50 ng/ml) for 6 h, and the conditioned medium was used to treat the RAW 264.7 macrophages for 24 h. IL-6 secretion by the macrophages was measured by ELISA. Each value represents the mean ± SD.

### Discussion

PGE2 plays a critical role in both acute and chronic inflammation. The most compelling evidence for the direct role of PGE2 in inflammation came from studies in animal models of inflammation. Selective neutralization of PGE2, in an animal model of Carrageenan-induced paw inflammation, prevented tissue edema and hyperalgesia in affected paws (28). Furthermore, in an adjuvant-induced arthritis model, administration of neutralizing PGE2 antibody reversed edema in the affected paw (28). These observations clearly demonstrate that PGE2 is necessary for the progression of acute and chronic inflammatory conditions in vivo. Several reports have shown that ω-3 fatty acid supplementation can lead to an increased synthesis of 3-series PG in vivo; however, the cellular effects of increasing the synthesis of 3-series PG have not been investigated. Moreover, very few studies have determined the effect of PGE3 on inflammatory processes. In one study, PGE3 had 67% lower oedemogenic effect compared with PGE2 in mice (29); however, the molecular mechanism by which these structurally similar molecules cause differential effects has not been determined.

We have shown that fish oil supplementation can significantly increase the concentration of EPA and DHA in plasma and tissues, thereby affecting the ω-3/ω-6 ratios (30, 31). EPA and DHA can compete with AA and reduce substrate availability for 2-series PG synthesis (25). Our data demonstrating that PGE2 synthesis is affected by altering the fatty acid composition of the membranes (Fig. 5A) are consistent with the idea that PG production can be modulated by dietary manipulation of PUFA.

The COX-2 gene is quiescent in most cells, including fibroblasts and macrophages. However, transcription of the COX-2 gene is dramatically activated by a wide variety of ligands, in many cell types (reviewed in ref. 32). In a number of cell and animal models, induction of COX-2 has been shown to promote cell growth, inhibit apoptosis, and enhance cell motility and adhesion. Interestingly, PGE3 has been shown to amplify its own production by inducing COX-2 expression in various cells (10, 14). Four PGE2 receptor subtypes, EP1, EP2, EP3, and EP4 (EP, endorphin), have been identified (33). By using EP-deficient mutant mice, it has been shown that PGE2 signaling is most likely transduced through the G protein-coupled EP2 receptor, which stimulates more production of PGE2 by boosting COX-2 expression through a positive-feedback mechanism (34). Thus, the PGE2 signal through the EP2 receptor may contribute to COX-2 effects observed in chronic inflammatory conditions and in colon tumors from both human and animal models (34).

The effect of PGE3 on COX-2 expression has not been studied. In fact, we originally hypothesized that COX metabolites derived from ω-3 PUFAs such as PGE3, may lack inflammatory and mitogenic properties due to their inability to induce COX-2 expression. Interestingly, although PGE2 is not mitogenic to NIH 3T3 cells, to our surprise, we found that both PGE2 and PGE3 induced COX-2 mRNA via similar signaling mechanisms (Fig. 4). However, at similar concentrations, PGE3 was a weak inducer of COX-2 compared with PGE2. Thus, it seems that although PGE2-mediated COX-2 expression can cause a positive-feedback mechanism of COX-2 induction and PGE2 synthesis (34), PGE3-mediated COX-2 expression can result in a negative-feedback mechanism and may decrease COX-2 induction and PGE2 synthesis. Thus, manipulating PG synthesis could attenuate the induction of COX-2 and the inflammatory response by dietary fatty acids. We have recently shown that a short-term dietary intervention with fish oil supplements in men with prostate cancer leads to a significant increase in the ω-3/ω-6 fatty acid ratios in plasma and adipose tissue (31). Furthermore, although this study included only a small number of subjects, COX-2 expression and PG production in prostatic tissue after the dietary intervention were significantly reduced in some patients (31). The potential for this diet to prevent the development and progression of prostate cancer by way of altered
COX-2 expression and PG production in prostatic tissue is under further study.

A number of studies have implicated a positive association between endogenous PGE\(_2\) production and release of inflammatory cytokine IL-6 (27, 35–37). Because macrophages are a major source of PGE\(_2\) during inflammation and because they have receptors for and respond to PGE\(_2\), we conducted experiments to see whether exogenous PGE\(_2\) could also play a role in IL-6 synthesis in macrophages. Our data show that macrophages respond to exogenous PGE\(_3\) by synthesizing IL-6 albeit to a far lesser extent than PGE\(_2\), suggesting that PGE\(_3\) is less effective than PGE\(_2\) in eliciting inflammatory cytokine production by the macrophages. Our studies suggest that COX-dependent conversion of \(\omega-6\) PUFA in inflamed or diseased tissues may result in a subdued inflammatory cytokine production compared with COX-dependent conversion of \(\omega-6\) PUFA.

What is the mechanism for the observed differences between the two structurally similar PG? One explanation for the observed results may be that PGE\(_3\) is promiscuous and may act through other EP receptors. We have recently completed a collaborative study with Scientists from The Merck Frost Centre for Therapeutic Research in Montreal, Canada, in which we tested the affinities of PGE\(_2\) and PGE\(_3\) toward all four human EP receptors (EP1, EP2, EP3-III, and EP4). Interestingly in these studies, \(K_i\) values for PGE\(_3\) were slightly higher than those for PGE\(_2\) for all four EP receptors (data not shown), suggesting that the differential effects between the two structurally similar PG are not due to promiscuous receptor usage but rather may be, in part, due to the differences in their affinities for the EP receptors. Alternatively, another explanation for the observed results may be that PGE\(_2\) and PGE\(_3\) have short but significantly different enough half-lives, which could lead to apparent differences in their effects.

We conclude that (i) PGE\(_3\), unlike PGE\(_2\), is not mitogenic to NIH 3T3 fibroblasts; (ii) PGE\(_2\) and PGE\(_3\) regulate transcription of the COX-2 gene in NIH 3T3 fibroblasts via similar signaling mechanisms; (iii) PGE\(_2\) and PGE\(_3\) both induce the secretion of IL-6 protein in RAW 264.7 macrophages; and (iv) the differential effects of PGE\(_2\) and PGE\(_3\) on cell proliferation and inflammation do not depend on their ability to induce COX-2 and IL-6 but rather may depend on the degree of COX-2 and IL-6 induction. Finally, our data suggest that successful replacement of \(\omega-6\) PUFA with \(\omega-3\) PUFA in cell membranes can result in a decreased cellular response to mitogenic and inflammatory stimuli.

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