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Inhibition of inducible prostaglandin E_2 synthase by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and polyunsaturated fatty acids

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Abstract

Prostaglandin E_2 synthase (PGE synthase) is one of the membrane-associated proteins in the eicosanoid and glutathione metabolism (MAPEG) family of microsomal enzymes and constitutes a novel inducible enzyme involved in inflammation and pyretic responses. We report, using a reversed-phase HPLC assay for the production of tritiated prostaglandin E_2 (PGE_2) by membranes from cells overexpressing human microsomal PGE synthase, that PGE synthase activity is inhibited effectively by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and arachidonic acid. The anti-inflammatory compound 15-deoxy-PGJ_2 was considerably more potent at inhibiting PGE synthase ($IC_{50} = 0.3$ μM) than the closely related PGJ_2 or Δ^{12}-PGJ_2, or the reaction product PGE_2. Arachidonic acid, docosahexaenoic acid, and eicosapentaenoic acid inhibited PGE synthase with a similar potency ($IC_{50} = 0.3$ μM) and were more potent inhibitors than various fatty acid analogues. The present results on the inducible PGE synthase extend observations on the ability to bind arachidonic acid to another member of the MAPEG family, and also suggest a novel mechanism of action for the anti-inflammatory effects of DHA, EPA, and 15-deoxy-PGJ_2. © 2002 Published by Elsevier Science Inc.

Keywords: Arachidonate; Prostaglandins; Inflammation; PGE synthase; MAPEG; 15-Deoxy-prostaglandin J_2

1. Introduction

PGE_2 has been shown to be a potent mediator of pain and inflammation [1–4] and has been implicated in the development of pyresis [5]. The synthesis of PGE_2 involves either cyclooxygenase-1 or the inducible cyclooxygenase-2, which convert arachidonic acid into PGH_2, followed by the conversion of PGH_2 to PGE_2 by cytosolic [6,7] or membrane-associated [8–12] PGE synthase. A membrane-associated, glutathione-dependent PGE synthase [8–12] has been shown recently to be up-regulated in cell lines and in macrophages following proinflammatory stimuli [9–11]. Induction of PGE synthase RNA was also reported in astrocytes treated with β-amyloid [8]. The inducible PGE synthase was found to couple better with cyclooxygenase-2 than cyclooxygenase-1 for the production of PGE_2 when co-transfected into mammalian cells [10]. In addition, the mRNA for the inducible PGE synthase was found to be increased in several rat tissues following the administration of lipopolysaccharide *in vivo* [10,12]. A marked increase in the production of the inducible PGE synthase at both the RNA and protein levels has also been observed in the rat adjuvant arthritis model [12], providing further support for a role of this enzyme in inflammatory responses. Therefore, PGE synthase appears to represent a major enzyme involved in cyclooxygenase-2-mediated PGE_2 production and constitutes a potential target for therapeutic intervention. Inducible PGE synthase has been identified to be a member of the recently identified MAPEG superfamily, which includes 5-lipoxygenase activating protein (FLAP) and LTC_4 synthase [13]. The homology between the members of the MAPEG family is reinforced by evidence of pharmacological cross-reactivity between these proteins [14,15], including the inhibition of LTC_4 synthase [16] and of PGE synthase [12] by the FLAP

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Abbreviations: $AACOCF_3$, arachidonyl trifluoromethyl ketone; AA-MeO, arachidonyl methyl ester; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EDA, eicosadienoic acid; FLAP, 5-lipoxygenase activating protein; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; MAFP, methyl arachidonyl fluorophosphonate; MAPEG, membrane-associated proteins in eicosanoid and glutathione metabolism; PG, prostaglandin; PGE synthase, prostaglandin E_2 synthase; PUFAs, polyunsaturated fatty acids; TXB_2, thromboxane B_2; 15-deoxy-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2.

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inhibitor MK-886. In the present report, we show that the activity of the inducible PGE synthase is also sensitive to inhibition by arachidonic acid and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-deoxy-PGJ_2).

2. Materials and methods

2.1. Materials

Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection. Cell culture medium, serum, antibiotics, and lipofectamine were purchased from Life Technologies. Restriction enzymes, ligase, and complete protease cocktail were obtained from Boehringer Mannheim. Tritiated [^{3}H]-PGH_2 (20 μCi/100 μL), a partially purified PGE synthase antibody, arachidonic acid, 5,6-dihydro-arachidonic acid, eicosapentaenoic acid (EPA), 11,14-EDA, 15(*S*)-HETE, U-51605, U-44069, 5-*trans*-U-44069, U-46619, 5-*trans*-U-46619, PGE_2, 15(*R*)-PGE_2, and PGE_3 were purchased from the Cayman Chemical Co., and 15-deoxy-PGJ_2 was purchased from the Calbiochem–Novabiochem Corp. All other fatty acids and prostaglandin (PG) were purchased from BIOMOL Research Laboratories Inc. Stannous chloride was obtained from BDH, and Western blot chemiluminescence reagent (Renaissance®) was obtained from NEN Life Science Products.

2.2. Cloning and expression of human PGE synthase

The cDNA encoding for human PGE synthase (GenBank Accession Number AF027740) was subcloned into the pcDNA 3.1(+) expression vector (Invitrogen) using the *Eco*RI–*Not*I restriction sites. The clone was sequenced using an Applied Biosystems 373A automated sequencer and dye terminator reactions as described by the protocol of the manufacturer and was subsequently transfected into CHO-K1 cells using Lipofectamine 2000. Both mock (pcDNA 3.1 vector alone) and human PGE synthase in pcDNA 3.1 transfected cells were prepared in a similar fashion. Briefly, cells were harvested 24 hr post-transfection, washed twice in 1× Dulbecco's phosphate-buffered saline, and resuspended in 15 mM Tris–HCl (pH 8.0), 0.25 M sucrose, 0.1 mM EDTA, and 1 mM glutathione in the presence of 1× complete cocktail of protease inhibitors. Resuspended cells were sonicated four times for 30 s at 4° using a Cole Parmer 4710 Ultrasonic Homogenizer at 70% duty cycle. Disrupted cells were subjected to centrifugation at 5000 *g* for 10 min and the resulting supernatant at 100,000 *g* for 1.5 hr at 4°. The 100,000 *g* membrane pellet (microsomal fraction) was resuspended in 10 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM EDTA, and 1 mM reduced glutathione. Expression of human PGE synthase was confirmed by Western blotting using a polyclonal PGE synthase antisera raised to residues 59–75 of human PGE synthase. Protein concentrations were determined using the Coomassie protein assay (Pierce) as described by the protocol of the manufacturer.

2.3. Measurement of PGE synthase activity

Microsomal PGE synthase activity was measured using [^{3}H]-PGH_2 as substrate and reversed-phase HPLC to quantitate the production of radiolabeled PGE_2 [12]. Incubation mixtures contained 100 mM potassium phosphate (pH 7.0), 2.5 mM glutathione, and 1 μg of protein (either from mock or human PGE synthase microsomal preparations). Reactions were performed using a final volume of 100 μL in 1.5 mL polypropylene tubes and at room temperature. Reactions were initiated by the addition of [^{3}H]-PGH_2 in ethanol (10 nCi) to obtain a final concentration of 1 μM, unless stated otherwise. The final concentration of ethanol in each reaction never exceeded 3% (v/v). For the inhibition studies, fatty acids and PGs were preincubated with PGE synthase for 15 min prior to the addition of substrate. Under these assay conditions, PGE synthase activity was determined to be linear for no longer than 45 s. To ensure that inhibition of PGE synthase activity is measured within the linear range, each reaction was quenched 30 s following the addition of substrate by the addition of 100 μL of 2.5 mg/mL of $SnCl_2$. The addition of $SnCl_2$ effectively converts all unreacted PGH_2 into $PGF_{2\alpha}$ to avoid non-enzymatic conversion to PGE_2 during sample processing. Then excess $SnCl_2$ precipitate was removed by centrifugation (15,000 *g* for 1 min at room temperature), followed by the addition to each sample of 1 μg of carrier unlabeled $PGF_{2\alpha}$ and PGE_2. A Waters 625 LC system was equipped with a C_{18} (3.9 mm × 150 mm) column equilibrated with 34% acetonitrile in water and 0.1% acetic acid (flow rate of 1 mL/min). Using the HPLC conditions, tritiated $PGF_{2\alpha}$ and PGE_2 were found to have retention times of 4.3 and 5.3 min, respectively (Fig. 1). Due to the inherent instability of PGH_2, a 10–20% conversion of the substrate to PGE_2 was observed in all mock reactions. Therefore, to determine the amount of [^{3}H]-PGE_2 formed enzymatically, all integrated peak areas for [^{3}H]-PGE_2 were subtracted by the background levels of [^{3}H]-PGE_2 found in the mock reactions. Percent inhibition of PGE synthase activity was then calculated using the corrected integrated peak areas for [^{3}H]-PGE_2 from reactions containing inhibitor and from control reactions with no inhibitor present.

3. Results

3.1. Inhibitory effects of PUFAs on PGE synthase activity

PGE synthase activity was quantitated using an assay based on the conversion of tritiated PGH_2 to PGE_2 and using stannous chloride to terminate the reaction so as to reduce unreacted PGH_2 to $PGF_{2\alpha}$ prior to HPLC analysis.

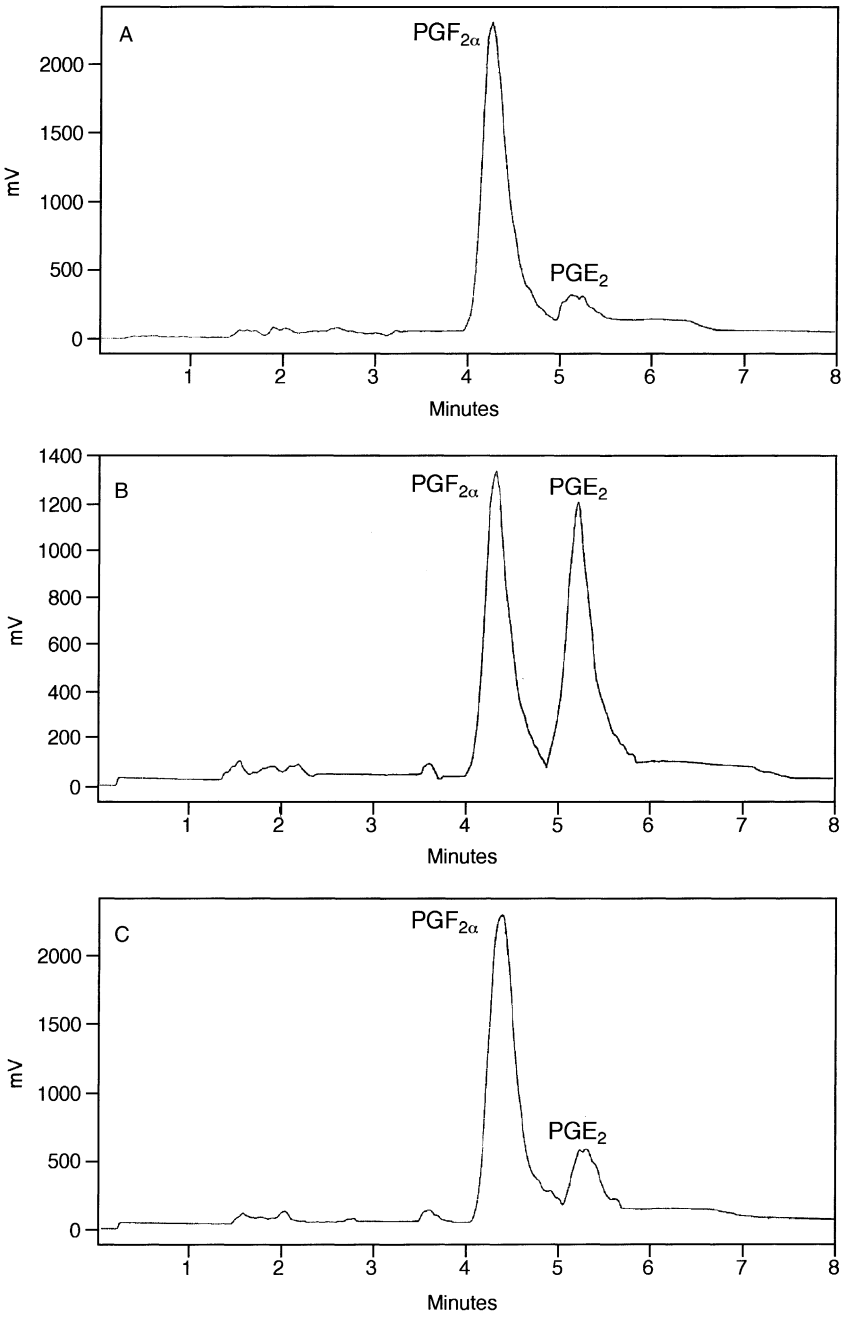

Fig. 1. HPLC chromatograms of the radiolabeled reaction products from the PGE synthase assay and inhibitory effects of arachidonic acid. Membrane preparations from mock-transfected cells (A) or from transfected cells overexpressing PGE synthase (B and C) were incubated for 30 s with [^{3}H]-PGH_2 before stopping the reaction with stannous chloride and HPLC analysis. The effect of the 15 min preincubation in the presence of 10 μM arachidonic acid is shown in panel C. Each experiment was performed in duplicate; data from one representative experiment are shown.

Typical chromatograms of the resolution of the reaction products are shown in Fig. 1 for membrane preparations from mock-transfected cells (Fig. 1A) or from cells expressing the inducible PGE synthase (Fig. 1B). Fig. 1C shows that the addition of 10 μM arachidonic acid strongly inhibited the production of PGE_2 by the synthase to a level similar to that observed for mock membranes. This assay was used to investigate the effects of polyunsaturated fatty acids (PUFAs) and various eicosanoids on the activity of PGE synthase. PGE synthase activity was inhibited by arachidonic acid and EPA with an IC_{50} value of 0.3 μM, as compared with IC_{50} values of 2 and 30 μM for palmitic acid and 6-heptenoic acid, respectively (Fig. 2). When arachidonic acid was compared to its analogues (Fig. 3), the inhibition of PGE synthase was reduced significantly upon the replacement of the free acid with a arachidonyl methyl ester (AAMeO), methyl arachidonyl fluorophosphonate (MAFP), or arachidonyl trifluoromethyl ketone (AACO-CF_3). The $AACOCF_3$ caused inhibition at higher concentrations with an approximate IC_{50} of 50 μM (data not shown).

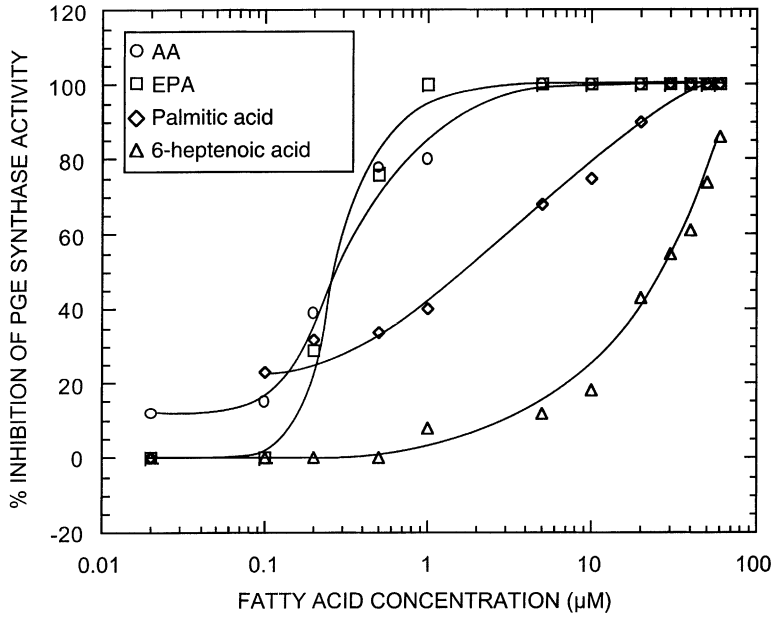

Fig. 2. Inhibition of PGE synthase by fatty acids. Each fatty acid was preincubated at the indicated concentrations for 15 min before measurement of PGE synthase activity. Results are presented as percent inhibition (average of duplicates) of the control reaction for (○) arachidonic acid (AA), (□) eicosapentaenoic acid (EPA), (◇) palmitic acid, and (△) 6-heptenoic acid.

Furthermore, 15(*S*)-HETE was less efficient than arachidonic acid in inhibiting PGE synthase activity but was more active than analogues lacking the free acid (Fig. 3). No reaction product could be detected by HPLC in incubation of the PGE synthase with ^{14}C-labeled arachidonic acid, indicating that arachidonic acid acts as an inhibitor of PGE synthase rather than as a substrate.

3.2. Effects of stable PGH_2 analogues and various prostaglandins on PGE synthase activity

Several PGs and close analogues of the PGH_2 substrate were also assayed for their effects on PGE synthase activity. U-44069 and U-46619 are essentially identical to PGH_2 except that each oxygen of the endoperoxide is

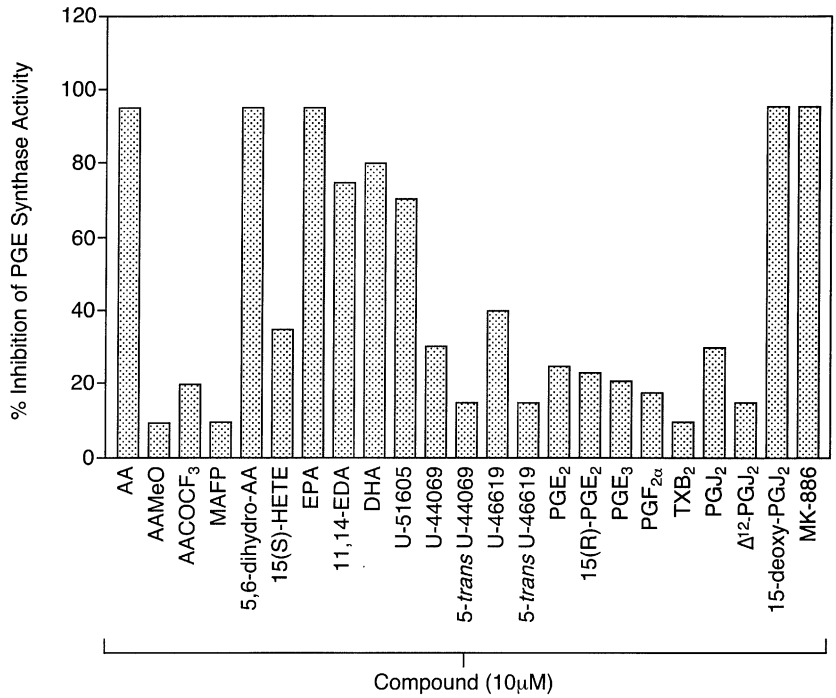

Fig. 3. Inhibition of PGE synthase activity by PUFAs and prostaglandins. The effect of each compound was measured at a concentration of 10 μM and using a 15 min preincubation before initiation of the reaction with the PGH_2 substrate. The percent inhibition of PGE synthase activity (average of two determinations) is shown for each of the compounds tested.

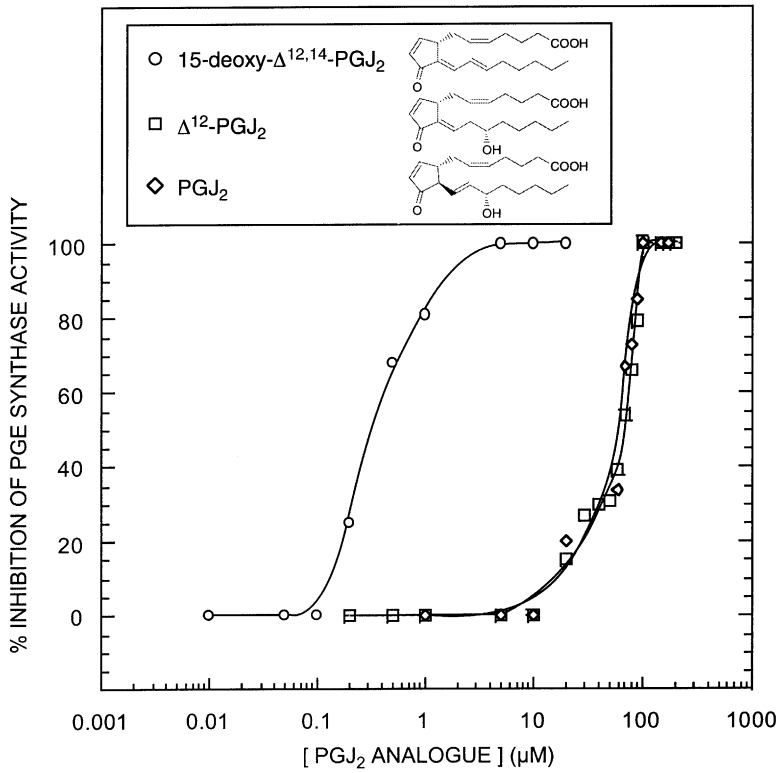

Fig. 4. Inhibition of PGE synthase activity by prostaglandins of the J_2 series. Prostaglandin J_2 and its analogues were preincubated at the indicated concentrations for 15 min before measurement of PGE synthase activity. Results are presented as percent inhibition of the control reaction (average of two determinations) for (○) 15-deoxy-$\Delta^{12,14}$-PGJ_2, (□) Δ^{12}-PGJ_2, and (◇) PGJ_2. The IC_{50} values for 15-deoxy-$\Delta^{12,14}$-PGJ_2, Δ^{12}-PGJ_2, and PGJ_2 are 0.3, 64, and 55 μM, respectively.

sequentially replaced by a methylene group. These stable analogues caused only low levels of inhibition of PGE synthase (<30%) at 10 μM (Fig. 3) and thus were considerably less potent inhibitors than PUFAs. U-51605, where the endoperoxide of PGH_2 is replaced by a diazo group, showed superior inhibitory activity when compared with either U-44069 or U-46619 with 70% inhibition at 10 μM. Interestingly, U-51605 also lacks the highly conserved hydroxy group at C_{15} found among most PGs. PGE_2, 15(*R*)-PGE_2, PGE_3, $PGF_{2\alpha}$, thromboxane B_2 (TXB_2), PGJ_2, and Δ^{12}-PGJ_2 had little effect (<30%) when tested at a concentration of 10 μM. The lack of inhibition by PGE_2 suggests that the reaction is not sensitive to product inhibition under the current assay conditions.

3.3. Inhibition of PGE synthase by 15-deoxy-PGJ_2

Among the various PGs tested (Fig. 3), 15-deoxy-PGJ_2 was found to be the most efficient at inhibiting PGE synthase. To directly compare the effect of the structurally related compounds PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-PGJ_2, concentration-dependence curves for the inhibition of PGE synthase were generated (Fig. 4). The inhibition was highly selective for 15-deoxy-PGJ_2 with an IC_{50} of 0.3 μM, more than two orders of magnitude lower than for Δ^{12}-PGJ_2 and PGJ_2 (IC_{50} values of 64 and 55 μM, respectively).

The inhibitory effects of 15-deoxy-PGJ_2 and arachidonic acid were found to be rapid, and similar potencies were observed when the preincubation was reduced from 15 min to 15 s. In addition, the effects of these compounds were only marginally shifted when the concentration of PGH_2 substrate was increased from 1 to 30 μM, in agreement with the lack of saturation of the reaction over this range of substrate concentrations (data not shown).

4. Discussion

The novel findings of this study are that PUFAs, including arachidonic acid, EPA, and docosahexaenoic acid (DHA), as well as the hydrophobic PG 15-deoxy-PGJ_2, are potent inhibitors of the inducible microsomal PGE synthase with IC_{50} values in the submicromolar range. LTC_4 synthase, FLAP, and PGE synthase are members of the MAPEG superfamily [13]. It has been established previously that FLAP lacks enzymatic activity yet binds to arachidonic acid, suggesting that FLAP functions as a lipid transfer protein for the 5-lipoxygenase reaction [17,18]. Furthermore, inhibition studies of LTC_4 synthase suggest the presence of an arachidonic acid binding site on this enzyme [14,15]. Hence, it appears that the ability to bind arachidonic acid is a conserved feature among several

members of the MAPEG superfamily. Whether binding to arachidonic acid renders PGE synthase capable of acting as a lipid transfer protein to other enzymes of the cyclooxygenase pathway in a manner similar to that proposed for FLAP and 5-lipoxygenase [17,18] still remains to be determined.

A feature of arachidonic acid that was found to be critical for its inhibitory activity is the free acid at C_1 since the methyl fluorophosphonate, trifluoromethyl ketone group, or methyl ester analogues were found to have a lower potency of inhibition. Hence, it may be reasonably assumed that the negative charge found in arachidonic acid, and presumably in PGs, is involved in the formation of a critical salt bridge with the surface of PGE synthase as has been proposed for hematopoietic PGD synthase when complexed to the PGH_2 substrate [19].

Among the various PGs tested, 15-deoxy-PGJ_2 was the most potent inhibitor of PGE synthase with an IC_{50} of 0.3 μM. This compound was shown to be a much more potent inhibitor of PGE synthase than either PGJ_2 or Δ^{12}-PGJ_2. 15-Deoxy-PGJ_2 can react with thiols to form Michael adducts [20], although it is not known whether the microsomal PGE synthase possesses an essential cysteine for activity. A major structural difference between 15-deoxy-PGJ_2 and its PGJ_2 analogues is the presence of a double bond between C_{14} and C_{15} instead of the hydroxyl group on C_{15} (see Fig. 4). Interestingly, U-51605 also lacks the hydroxyl group at C_{15} and was found to be a better inhibitor than most other PGs, whereas 15(*S*)-HETE was less potent than arachidonic acid. Taken together, these data indicate that the interaction between eicosanoids and PGE synthase requires the free carboxylic acid at C_1 and is reduced by the presence of the polar hydroxyl group at the C_{15} position.

Anti-inflammatory effects of 15-deoxy-PGJ_2 have been suggested based on its interaction with the peroxisome proliferator-activated receptor γ (PPARγ) [21,22]. The concentration of 15-deoxy-PGJ_2 reported for PPARγ binding and for PPARγ-mediated anti-inflammatory effects (0.5–10 μM) lies within a similar range to the IC_{50} value reported in this study for the inhibition of PGE synthase activity [23,24]. Therefore, the inhibition of inducible PGE synthase may represent a novel mechanism for the proposed anti-inflammatory effects of 15-deoxy-PGJ_2. PPARγ-independent anti-inflammatory effects have also been described, including the down-regulation of activated microglia via inhibition of NFκB transcriptional activity [25], cytokine production by monocytes [26], and inhibition of neutrophil adhesion and respiratory burst [27]. Furthermore, the anti-inflammatory effect of 15-deoxy-PGJ_2 for interleukin-1β-induced PGE_2 synthesis in rheumatoid synovial fibroblasts has been shown to be independent of PPARγ and to be associated with a negative feedback on the COX pathway [28]. Therefore, it would be of interest to determine whether the inhibition of PGE synthase by 15-deoxy-PGJ_2 could explain the effects observed in these systems. In the carrageenan-induced pleurisy, the generation of PGD_2 and 15-deoxy-PGJ_2 has been associated with the resolution of inflammation, and an exacerbation of a late phase of inflammation at 48 hr has been associated with the inhibition of their syntheses by the selective cyclooxygenase-2 inhibitor NS-398 [29]. Interestingly, this late phase of inflammation was associated with minimal PGE_2 synthesis [29]. Hence, inhibition of inducible PGE synthase by 15-deoxy-PGJ_2 could provide a mechanism for this anti-inflammatory PG and warrants further investigation. The anti-inflammatory activity of 15-deoxy-PGJ_2 has been demonstrated recently *in vivo*, following intraperitoneal administration of the compound in an adjuvant-induced arthritis model in rats [30]. Furthermore, the high potency of DHA and EPA as inhibitors of the inducible PGE synthase suggests a plausible mechanism for the demonstrated anti-inflammatory properties of these PUFAs in various animal models [31–34].

In conclusion, it has been determined that along with LTC_4 synthase [14–16] and FLAP [17,18], the capacity to bind arachidonic acid is conserved for the PGE synthase member of the MAPEG superfamily. The results from this study also suggest that the inhibition of the enzymatic activity of the inducible PGE synthase could explain some of the anti-inflammatory properties of DHA, EPA, and 15-deoxy-PGJ_2.

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