Stimulation of prostaglandin G/H synthase-2 expression by arachidonic acid monoxygenase product, 14,15-epoxyeicosatrienoic acid

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Abstract The relationship between arachidonic acid (AA) mobilization and transcription of immediate-early genes, particularly of prostaglandin G/H synthase-2 (PGHS-2), in intestinal crypt epithelial cells was analyzed. PGHS-2 mRNA and protein synthesis were stimulated by its own substrate, AA; actinomycin D, a transcription inhibitor, prevented the AA-induced increase in PGHS-2 mRNA. Eicosatetraynoic acid, an inhibitor of AA utilization, significantly reduced PGHS-2 mRNA synthesis elicited by AA. Inhibitors of cytochrome P450 monoxygenases, ketoconazole and miconazole, also prevented PGHS-2 mRNA synthesis in a dose-dependent manner. Phenyl chalcone oxide, an epoxide hydrolase inhibitor, potentiated AA-induced PGHS-2 mRNA synthesis. Of the four regioisomers of arachidonic acid epoxides, only 14,15-epoxyeicosatrienoic acid elicited the expression of PGHS-2 in intestinal crypt epithelial cells. This is the first direct evidence of stimulation of an immediate-early gene product, specifically PGHS-2, by an AA epoxygenase metabolite, 14,15-epoxyeicosatrienoic acid, as well as of a heterologous regulation of PGHS-2 synthesis by these monoxygenase products.

Key words: Prostaglandin G/H synthase-2; Prostaglandin synthesis; Arachidonic acid; Cytochrome P450 monoxygenase; Gene transcription; Epoxyeicosatrienoic acid

1. Introduction

Inflammatory mediators, growth factors and pro-oxidants, known to mobilize arachidonic acid (AA) from membrane phospholipids [1–3], have also been shown to stimulate the synthesis of prostaglandin G/H synthase-2 (PGHS-2), an inducible enzyme of the prostaglandin pathway of AA metabolism [3,4]. In colonic mucosa where moderate expression of PGHS-2 is normally detected [5], unregulated synthesis of PGHS-2 has been shown to render intestinal crypt cells refractory to programmed cell death [6], a predisposing event in colon carcinogenesis [7]. Increased phospholipase activity [8], higher AA content in membrane phospholipids [8,9] and overexpression of PGHS-2 in colonic tumors [8,10] coupled with lowered incidence of colorectal cancer with the use of non-steroidal anti-inflammatory drugs [11] pointed to a clinically significant relationship between AA metabolism and PGHS-2 expression. However, the role of AA per se in this process is not known.

AA can directly affect ion channels [12,13] but most AA undergoes oxidation through the cyclooxygenase, lipoxygenase and epoxygenase pathways to yield prostaglandins (PG), prostacyclin, thromboxane, leukotrienes, lipoxins and hydroxy-, hydroperoxy- and epoxy-fatty acids. Some products of these pathways have been shown to affect expression of immediate-early genes. For example, c-*fos* has been shown to be induced by PGE_2 [14], leukotriene B_4 [15] and epoxygenase metabolites [16]. However, only PGs have been reported to increase the expression of PGHS-2 in osteoclasts [17], whereas the role of the other two AA pathways, lipoxygenases and monoxygenases, in eliciting PGHS-2 transcription is not known. Here we report that AA epoxygenase products, specifically 14,15-epoxyeicosatrienoic acid (14,15-EET), induced the synthesis of PGHS-2 mRNA in intestinal epithelial cells. To our knowledge, this is the first report of a heterologous regulation of PGHS-2 synthesis by the cytochrome P450 epoxygenase products of AA.

2. Materials and methods

2.1. Materials

Polyclonal rabbit antibodies specific to PGHS-2 were generously provided by Drs. A.W. Ford-Hutchinson and G.P. O'Neill (Merck Frosst Center for Therapeutic Research, Pointe-Claire, Quebec). LY233569 was also a gift from Eli Lilly and Co. (Indianapolis, IN). The following reagents were purchased: peroxide-free AA, eicosatetraynoic acid (ETYA), methyl esters of (±)5,6-EET, (±)8,9-EET, (±)11,12-EET, (±)14,15-EET (Cayman Chemical, Ann Arbor, MI); [^{32}P]CTP (3000 Ci/mmol), enhanced chemiluminescence kit (Amersham Canada, Mississauga, Ont.); aprotinin, leupeptin (Boehringer Mannheim, Montreal, Que.); pGEM-4 plasmid vector, in vitro transcription kit (Promega, Madison, WI); protein assay and electrophoretic reagents (Bio-Rad, Mississauga, Ont.); guanidine isothiocyanate, proteinase K, RNase A, RNase T_1, restriction enzymes (BRL Life Technologies, Burlington, Ont.); rat β-actin riboprobe (Ambion Inc, Austin, TX); soybean trypsin inhibitor (type II-S), phenylmethylsulfonyl fluoride (PMSF), ketoconazole and miconazole, (Sigma, St. Louis, MO). All other chemicals were of analytical reagent grade and were purchased from either Sigma (St. Louis, MO) or ICN Biochemicals (Montreal, Que.).

2.2. Cell culture and drug treatments

Rat intestinal epithelial primary cells, IEC-18 (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics, penicillin (10 U/ml) and streptomycin (10 μg/ml) in a humidified atmosphere containing 5% CO_2 at 37°C. Confluent primary cell cultures of IEC-18 (passages 16–20) were rendered quiescent by maintaining them in DMEM containing 0.5% fetal bovine serum for 24 h. Drugs and other chemicals were added to the cultures as described in the figure legends.

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Abbreviations: PGHS, prostaglandin G/H synthase; ETYA, eicosatetraynoic acid; EET, epoxyeicosatrienoic acid; PG, prostaglandin; AA, arachidonic acid

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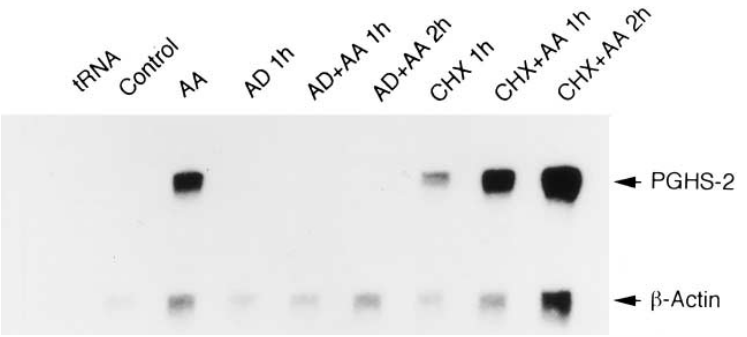

Fig. 1. Stimulation of PGHS-2 transcription by AA and the effects of transcription and translation inhibitors, respectively actinomycin D (AD) and cycloheximide (CHX). Quiescent IEC-18 cells were pretreated 20 min with drugs and exposed to 10 μM AA for the durations indicated. The antisense probes for rat PGHS-2 and β-actin comigrated at the same position (200 nt). The protected fragments for rat PGHS-2 and β-actin were 176 nt and 127 nt respectively. The relatively weaker signal of β-actin protected fragments was due to use of antisense probes of approximately 10-fold less specific activity. Autoradiographic exposures were for overnight.

2.3. Total RNA isolation

The monolayers of cells were washed once with ice-cold phosphate-buffered saline (PBS) and the total RNA was extracted by using the acid phenol guanidine isothiocyanate method [18]. The RNA was quantified by measuring the absorbance at 260 nm.

2.4. Preparation of rat PGHS-2 and RNase protection assays

Reverse transcription of total RNA isolated from serum-stimulated IEC-18 cells, followed by amplification of the cDNA using gene-specific primers for rat PGHS-2 [19] and Taq polymerase were conducted as described before [20]. The following primer pairs were used: rat PGHS-2: 5′-TGC CAC CTC TGC GAT GCT CTT CC-3′ and 5′-TTC TTG TCA GGA AAT CTC GGC G-3′. The partial cDNA of rat PGHS-2 (0.2 kbp) was cloned in pGEM-4 vector by blunt-end ligation. Multiple plasmid clones were sequenced to verify the nucleotide sequence of rat PGHS-2. The cDNA probe used for rat PGHS-2 spans across the first two exons so that the protected fragments in RNase protection assays represent the correctly spliced mRNA [21].

^{32}P-Labelled cRNA probes for rat PGHS-2 and rat β-actin (used as control for input RNA) were prepared using an in vitro transcription kit. Aliquots of the total RNAs were subjected to RNase protection assays according to a published protocol [22] with minor modifications. Briefly, 10 μg of total RNA was incubated for 1 h at 50°C with 5×10^4 cpm of cRNA probes in 20 μl of hybridization buffer (80% deionized formamide, 40 mM PIPES, pH 6.8, 1 mM EDTA and 0.4 M NaCl). The RNA hybrids were digested in 200 μl of digestion buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 0.3 M NaCl) containing ribonuclease A (10 μg/ml) and RNase T_1 (250 units/ml) for 30 min at 25°C. Proteinase K treatment followed by precipitation of protected fragments was conducted exactly as described [22]. The protected RNA fragments were resolved on urea-8% polyacrylamide gels and the bands were visualized by phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

2.5. Immunochemical detection of PGHS-2

Following stimulation by 10 μM AA, confluent monolayers of cells were washed twice in ice-cold PBS and lysed directly in ice-cold RIPA buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% sodium desoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, and 10 μg/ml each of leupeptin, aprotinin, soybean trypsin inhibitor, 0.2 mM PMSF). Homogenates were then centrifuged at $12\,000\times g$ for 10 min at 4°C. Protein concentration of the supernatant was determined using the Bio-Rad protein assay reagent. Aliquots of total protein (50 μg) were denatured in sample buffer (125 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 10% β-mercaptoethanol and 0.1 mg/ml bromophenol blue) for 15 min at room temperature and boiled for 5 min before loading on SDS-polyacrylamide gels. Electrophoretic transfer of the proteins to PVDF membranes, successive incubations with PGHS-2 specific antiserum and horseradish peroxidase-conjugated anti-rabbit IgG antibodies were conducted as described previously [20]. The immunoreactive bands were visualized by using the enhanced chemiluminescence kit as instructed by the manufacturer.

3. Results and discussion

Aberrant expression of PGHS-2 in intestinal epithelial cells is shown to protect the cells from apoptosis [7], an important step in colon carcinogenesis [8,10]. Alterations in fatty acid metabolism, a consequence of which may be increased availability of AA in colonic tumors [9], may have a direct bearing upon the unregulated expression of PGHS-2. Besides prostaglandins (PG), specifically PGE_2 [17], none of the other products of AA have been shown to stimulate PGHS-2 expression. In order to find out if AA and its metabolites caused a stimulation of PGHS-2 synthesis in colonic epithelial cells, we treated quiescent IEC-18 cells with AA in the presence of inhibitors of AA metabolism and determined the levels of PGHS-2 mRNA by RNase protection assay.

Direct addition of micromolar concentrations of AA to intestinal crypt primary cells caused a rapid increase in the abundance of PGHS-2 mRNA (Fig. 1), commensurate with an immediate-early gene response typical of PGHS-2 [23]. This accumulation of PGHS-2 mRNA was abolished when the cells were treated with transcription inhibitor, actinomycin D (Fig. 1). Similarly, as expected for immediate-early genes, inhibition of protein synthesis with cycloheximide, prevented the degradation of PGHS-2 mRNA and as a result, superinduced PGHS-2 mRNA. Thus it appeared that the stimulatory effect of AA upon PGHS-2 mRNA abundance was at the level of gene transcription. In agreement with elevated PGHS-

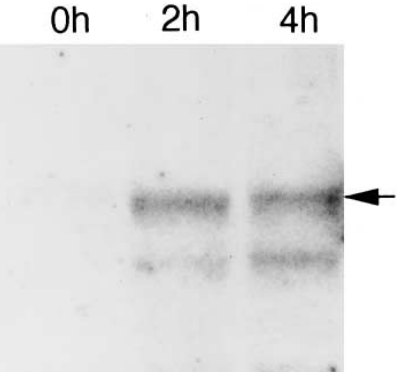

Fig. 2. Immunoblot analysis of PGHS-2 protein (indicated by an arrow) in quiescent IEC-18 cells treated with 10 μM AA for 2 and 4 h (the doublet appearance of the 70 kDa PGHS-2 may be due to endogenous proteolysis).

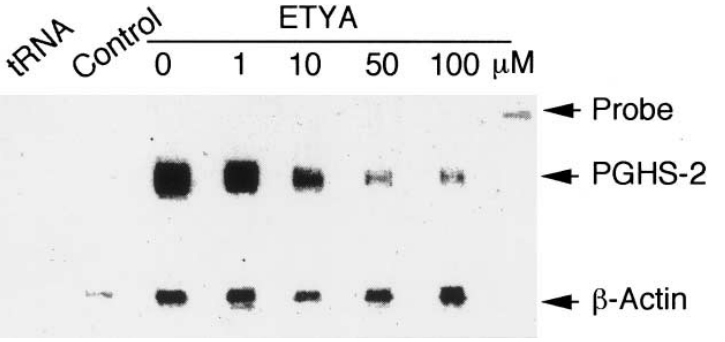

Fig. 3. Dose response of the AA utilization inhibitor, ETYA. Cells were pretreated 20 min with the indicated concentrations of the inhibitor and exposed for 1 h to 10 μM AA. The autoradiographic exposure was for overnight.

2 mRNA levels, immunoblot analysis of detergent-lysates of IEC-18 cells treated with AA for 2 h revealed the appearance of 70 kDa PGHS-2 immunoreactive band (Fig. 2).

AA has been shown to exert direct effects independent of its metabolism on such targets as ion channels [12,13] and protein tyrosine kinase activity [24]. In order to find out if metabolism of AA is required for upregulation of PGHS-2 synthesis, quiescent IEC-18 cells were treated with eicosatetraynoic acid (ETYA), an inhibitor of AA utilization, in the presence of AA. ETYA reduced PGHS-2 mRNA abundance in a dose-dependent manner, suggesting that this response was specific to AA and mediated by its metabolites (Fig. 3). However, addition of prostaglandins (PGE_2, $PGF_{2\alpha}$, PGD_2 and PGI_2) or a thromboxane mimetic (U46619) to IEC-18 cells did not reproduce the effects of AA. Similarly, specific inhibitors of the cyclooxygenase (diclofenac and indomethacin) and lipoxygenase (caffeic acid and nordihydroguaiaretic acid) pathways of AA metabolism did not prevent AA-induced PGHS-2 mRNA synthesis (data not shown). Hence, the third pathway of AA utilization, mediated by the cytochrome P450 (CYP 450) monoxygenases which produces epoxyeicosatrienoic acids (EETs) and hydroxyeicosatrienoic acids (HETEs), seemed to mediate AA-induced PGHS-2 synthesis [25,26]. To test if CYP 450 monoxygenases are involved in the stimulation of PGHS-2 synthesis, quiescent IEC-18 cells were treated with ketoconazole and miconazole before the addition of AA. Both these monoxygenase blockers significantly reduced PGHS-2 mRNA synthesis (Fig. 4), thus revealing a role of the products of AA monoxygenase pathway in this process.

Of the numerous monoxygenase products of AA, EETs have been proposed to increase in response to various stimuli, promote mitogenesis and induce immediate-early genes, such as c-*fos* and Egr-1 [16]. Pretreating quiescent IEC-18 cells with phenyl chalcone oxide, an inhibitor of epoxide hydrolases [27], potentiated AA-induced PGHS-2 (another immediate-early gene) mRNA accumulation (Fig. 5, right), suggesting a role for EETs, but not their hydrolysis products, vicinal diols, in this process. Therefore we tested if EETs could reproduce AA-induced upregulation of PGHS-2 synthesis in IEC-18 cells. Indeed, the addition of (±)14,15-EET (Fig. 5, left) at concentrations comparable to AA, also caused a rapid increase in PGHS-2 mRNA transcription; but none of the other regioisomers of EETs were able to induce PGHS-2 mRNA synthesis (Fig. 5, left). These data support the involvement of EETs in AA-stimulated PGHS-2 synthesis; a role for monoxygenase-derived HETEs could not, however, be excluded.

It is worth noting that the concentration of (±)14,15-EET needed to elicit PGHS-2 transcription in IEC-18 cells was comparable to the concentrations of PGE_2 in osteoclasts [17] and to AA in inducing c-*fos* and Egr-1 in mesangial cells and fibroblasts [14,16]. It is of interest that oxidant stress which causes mobilization of AA [1–3] also increases EET production [28] and stimulates PGHS-2 synthesis [29]. Taken together, our observations imply a significant involvement of 14,15-EET as a mediator of AA-elicited PGHS-2 synthesis (possibly other immediate-early genes as well), in intestinal epithelial cells.

In conclusion, we here report the first direct evidence of stimulation of PGHS-2 synthesis by an AA epoxygenase metabolite, 14,15-EET. These results also suggest a novel regulation of PGHS-2 synthesis which can occur not only by its own product [17], PGE2 (autologous regulation), but also by the products of enzymes of other AA metabolic pathways (heterologous regulation), specifically epoxygenases (this study). This diverse regulation of PGHS-2 by AA metabolites,

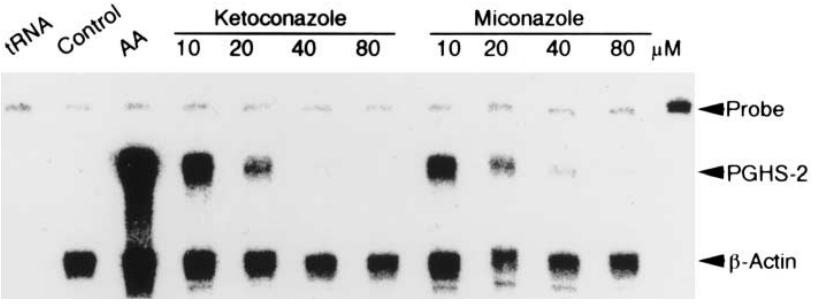

Fig. 4. Effects CYP 450 monoxygenase inhibitors, ketoconazole and miconazole, on PGHS-2 transcription at 1 h induced by 10 μM AA. Phosphorimager-derived image of the gel.

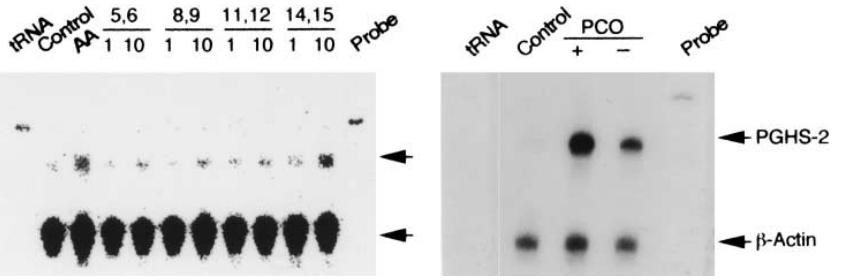

Fig. 5. Left: Stimulation of PGHS-2 transcription by epoxyeicosatrienoic acids. Effects of AA (10 μM and (±)5,6-, (±)8,9-, (±)11,12- and (±)14,15-EET methyl esters at concentrations of 1 and 10 μM on PGHS-2 mRNA levels (phosphorimager-derived image) after 1 h of treatment. Right: Potentiaton of AA-induced PGHS-2 mRNA accumulation by an epoxide hydrolase inhibitor, phenyl chalcone oxide (PCO). Quiescent cells were pretreated for 10 min with 1 μM PCO before adding 10 μM AA and PGHS-2 mRNA levels determined after 1 h of treatment. Autoradiography was for overnight.

particularly in view of its anti-apoptotic role, may be relevant in oxidant stress, inflammation and oncogenesis.

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