Prostaglandin E_2 regulation of cyclooxygenase expression in keratinocytes is mediated via cyclic nucleotide-linked prostaglandin receptors

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Abstract **Inflammatory responses are thought to be mediated in part by the prostaglandins derived from arachidonic acid (AA) by the action of prostaglandin H synthase, also referred to as cyclooxygenase (COX). The mitogen-inducible isoform, COX-2, is over-expressed in numerous chronic inflammatory disease conditions and in neoplasms from both human and experimental animal models. COX-1 expression, on the other hand, has been referred to as constitutive or non-inducible. In this study, we present evidence demonstrating autoregulation of prostaglandin (PG) production by the PGs themselves and their precursor, AA. We observed that AA and PGs induced COX-2, as well as COX-1, expression in cultured murine keratinocytes approximately 3 h after treatment. In primary keratinocytes transiently transfected with a full-length COX-2 promoter linked to a luciferase reporter gene, we observed enhanced transcription by AA, PGE_2, and the other prostaglandins. Forskolin, a known activator of adenylate cyclase, and dibutryl-cAMP, a cAMP analog, induced COX-1 and COX-2 mRNA, suggesting that cAMP is a second messenger for COX expression. SQ 22536, an adenylate cyclase inhibitor, inhibited COX-2 mRNA induction by PGE_2 in a dose-dependent manner suggesting that PGE_2-induced expression may be through one of the cAMP-linked PGE_2 receptors. The results of this study demonstrate that both COX-1 and COX-2 are inducible. Further, both COX isoforms can be up-regulated by their products, the PGs, and this autoregulation probably occurs via prostaglandin receptors linked to a cAMP signal transduction pathway.**—Maldve, R. E., Y. Kim, S. J. Muga, and S. M. Fischer. **Prostaglandin E_2 regulation of cyclooxygenase expression in keratinocytes is mediated via cyclic nucleotide-linked prostaglandin receptors.** ***J. Lipid Res.*** **2000.** 41: **873–881.**

Supplementary key words prostaglandins • cyclooxygenase • signal transduction

Prostaglandin production is catalyzed by cyclooxygenase (COX) from arachidonic acid (AA) through a two-step process involving distinct cyclooxygenase and peroxidase activities (1). Two isoforms of COX have been identified and characterized: COX-1 is constitutively expressed in many tissues and cell types and is presumed to be involved in maintaining cell homeostasis, whereas COX-2 is transiently induced after mitogenic or inflammatory stimuli, implicating COX-2 in the production of prostaglandins involved in inflammatory responses (2, 3).

With regard to epidermal keratinocytes, there are a number of observations that suggest that PG synthesis is not only highly regulated but also that it is required for skin tumor development. Exposure of skin to ultraviolet (UV) irradiation or of keratinocytes to diverse classes of chemical tumor promoters induces the expression of COX-2 and elevates the synthesis of PGE_2 (4–6). The requirement of high PGE_2 levels for skin tumor development is suggested by studies in which inhibitors of COX had strong chemopreventive activity. Indomethacin, a nonsteroidal anti-inflammatory drug, with both COX-1 and COX-2 inhibitory activity, reduced tumor multiplicity significantly in both chemical and UV carcinogenesis models (4, 5). However, the induced expression of COX-2 but not COX-1 by tumor promoters or UV suggests that COX-2 is responsible for the elevation in PGE_2 levels. Recently, we showed that a COX-2 selective inhibitor, celecoxib, both reduced PGE_2 synthesis and significantly inhibited UV-elicited skin tumor development (4). Additionally, COX-2 is constitutively up-regulated in tumors of various types, including skin (4, 5). Collectively, these and other studies suggest that COX-2 expression is highly regulated; an understanding of its regulation could potentially offer targets for the prevention of a number of pathologies.

Prostaglandins are novel ligands for receptor-mediated pathways that may contribute to prolonged activation of biological responses such as cell growth and differentia-

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; EMEM, Eagle's minimal essential media; EPA, eicosapentaenoic acid; INDO, indomethacin; LA, linoleic acid; NDGA, nordihydroguaiaretic acid; PPAR, peroxisome proliferator-activated receptor; PG, prostaglandin; SA, stearic acid.

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tion (7, 8). The rapid metabolic breakdown of prostaglandins suggests that they may act as transient autocrine or paracrine factors to modulate the action or synthesis of other second messengers. It is now well established that the major prostaglandins bind to specific receptor subtypes. For example, PGE_2 is the ligand for four receptor isoforms, termed EP1, EP2, EP3, and EP4, that directly modulate intracellular levels of inositol phosphate or cAMP (reviewed in ref. 9). Additionally, AA and its COX metabolites have been shown to activate several targets, including NF-κB, protein kinase C, Ras-GAP, and peroxisomal proliferator-activated receptors (PPARs), suggesting additional mechanisms by which eicosanoids may modulate gene expression (10–13).

The possibility that prostaglandins could exert regulatory feedback actions via the expression of their own enzymes has been demonstrated recently in osteoblastic cells (14–16), human osteosarcoma cells (17), macrophages (18), and prostate carcinoma cells (19). In part, the increases in COX-2 expression by PGE_2 were mediated by cAMP (15, 18) or via increases in cytosolic phospholipase A_2 (14).

In this study we evaluated the regulation of COX-2 in keratinocytes by various eicosanoids and determined that AA and its major prostaglandin metabolites can increase COX-2, as well as COX-1, expression. In addition, we present evidence that COX-2 expression can be regulated by activation of a PGE_2 receptor linked to a cAMP/PKA signal cascade.

MATERIALS AND METHODS

Stearic acid, indomethacin, nordihydroguaiaretic acid (NDGA), dibutryl cAMP, and forskolin were products of Sigma Chemical Co. (St. Louis, MO). Arachidonic acid, linoleic acid, and the prostaglandin metabolites (PGE_2, PGD_2, $PGF_{2\alpha}$) were purchased from Cayman Chemical Co. (Ann Arbor, MI). SQ 22536 was the product of Calbiochem (La Jolla, CA). AH-6809 was obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). NS 398 was a generous gift from Dr. N. Futaki (Taisho Pharmaceutical Co., Saitama, Japan). The luciferase reporter vector (pTIS10L) containing the promoter region of the mouse COX-2 gene was provided by Dr. H. Herschman (University of California, Los Angeles). An internal deletion of the cAMP response element was made in this construct, referred to as d-68/-51, as previously described (20). TriReagent™ is the product of Molecular Research Center (Cincinnati, OH). [α-^{32}P]dCTP (3000 Ci/μmol) and [1-^{14}C]arachidonic acid (specific activity, 55–60 μCi/mmol) were purchased from DuPont NEN (Boston, MA). The random primed DNA labeling kit is the product of Ambion (Austin, TX). The cDNA probes for Northern analysis of COX-1 and COX-2 were products of Oxford Biomedical Research (Oxford, MI). The cDNA for 7S RNA was obtained from Dr. A. Balmain (University of Glasgow, Glasgow, UK). The cAMP enzymeimmunoassay was purchased from Amersham Corp. All chemicals for RNA isolation were of molecular biology grade and RNAse free. Solvents and other chemicals were of the highest quality available.

Cell culture system and mRNA isolation

Murine epidermal keratinocytes were harvested from newborn (24 h) SENCAR (SSIN) mice by trypsinization as described by Yuspa and Harris (20). Keratinocytes were plated in an enriched Waymouth's media (1.2 mm Ca^{2+}) containing 10% fetal bovine serum at a density of $5–7 \times 10^6$/100-mm dishes for Northern analysis, or 3×10^6 cells/35-mm dish for arachidonate metabolism analysis, and incubated at 37°C in 5% CO_2. The cells were allowed to attach for 2.5 h at which time the medium was replaced with Eagle's minimal essential media (EMEM; 0.05 mm Ca^{2+}) plus 8% chelexed serum but without added growth factors. The cultures were not refed for 48 h prior to treatment to ensure depletion of serum and growth factors. Upon treatment of the cultures, the medium was removed from the dish, stimuli and/or inhibitors were added to the medium, gently mixed and the treated medium was added back to the appropriate dish. At appropriate times, medium was removed, cells were rinsed with PBS and scraped into 2 ml TriReagent™ per 100 mm dish and stored in 15 ml polypropylene conical tubes at −80°C for later Northern analysis.

Northern analysis

Total cellular RNA was isolated by the protocol provided by TriReagent™. One 100-mm dish per treatment group was used. Ten micrograms of RNA per sample was denatured at 68°C for 10 min, electrophoresed through 1% agarose gels containing 0.65 m formaldehyde, and transferred onto a nylon membrane. The membrane was UV cross-linked and hybridized to random-primed ^{32}P-labeled cDNA probes for COX-1 or COX-2 (Oxford Biomedical Research, MI) in QuickHyb™ solution (Stratagene, La Jolla, CA) for 1 h at 68°C. After hybridization, the membranes were washed 2 times in a low stringency buffer (2× SSC and 0.1% SDS) for 15 min each at room temperature and once in a high stringency buffer (0.2× SSC and 0.1% SDS) for 30 min at temperatures recommended for each of the cDNA probes. Autoradiographs were prepared using Kodak XAR-5™ film with intensifying screens at −80°C. Control for loading was obtained by stripping the blots of the COX-1 and COX-2 probes and rehybridizing with a probe specific for the 7S cytoplasmic RNA, which is present in the same abundance in all epidermal tissues (21). Autoradiographs were scanned by densitometry and normalized to the 7S RNA. All experiments were performed at least twice; representative experiments are shown. The data were evaluated for statistical significance using Student's *t*-test for dependent samples.

Arachidonic acid release and prostaglandin synthesis

Media from 2-day serum-depleted 35-mm cultures were pooled and [^{14}C]arachidonic acid (0.10 μCi/ml media) was added. The dishes were refed with the radioactive media and incubated for 16 h. The following day, the 'hot' medium was removed and replaced with conditioned media containing the stimuli and/or the inhibitor. At designated times, 1-ml aliquots of media were removed from the treated cultures and extracted as previously described (22). Briefly, aliquots of media were acidified to pH 3 with 1 N HCl and extracted twice with 4 ml of ethyl acetate. The pooled extracts were dried under a N_2 stream, redissolved in 100 μl of ethyl acetate, and applied to TLC plates for analysis of PGE_2. The plates were developed with the organic phase of ethyl acetate–2, 2, 4-trimethylpentane–acetic acid–water 11:5:2:10 (v/v/v/v) (23). The separated bands were detected by staining with iodine vapors and autoradiography. The bands corresponding to an authentic PGE_2 standard were cut from the plate, placed in scintillation vials, and counted.

cAMP measurement

Scraped cells were precipitated with 6% trichloroacetic acid and homogenized for 30 sec. The supernatants (13,000 rpm) were extracted 4 times with 5 volumes of water-saturated diethyl ether, while the pellets were dissolved in 1 ml of 1 N NaOH for protein determinations. The aqueous extracts were dried under

nitrogen stream at 60°C and reconstituted in a suitable volume of assay buffer. The cAMP nucleotides in the samples were measured according to the non-acetylation method outlined in the manufacturer's instructions and quantitated by enzyme immunoassay.

Transient transfection of primary keratinocytes

Newborn keratinocytes were plated as described above in 35-mm dishes at a cell density that would reach 60% confluency in 18–24 h. For transfection, 4 μg luciferase-reporter construct and 1 μg of pRSVβgal and 10 μl lipofectin reagent (Life Technologies Inc.) per dish were incubated at room temperature for 15 min in serum- and antibiotic-free EMEM before addition to the cultures. Cells were incubated with the DNA/lipofectin mixture for 5 h after which time the serum-free medium was removed and the dishes were refed for 18 h with EMEM containing 0.05 mm Ca^{2+} plus 8% chelexed serum EMEM. Cells were treated with agents 24 h after transfection and were harvested according to the protocol provided with the chemiluminescence reporter assay kit (Galacto-Light; Tropix, Inc, Bedford, MA). Briefly, cells were scraped into 250 μl lysis solution (100 mm potassium phosphate, pH 7.8, 0.2% Triton X-100, 1 mm dithiothreitol) and cetrifuged at 12,000 *g* for 2 min. Twenty microliters of the supernatant was aliquoted in duplicate into 12 × 75 mm borosilicate tubes. Chemiluminescence activity was measured with a luminometer and values were expressed as the ratio of luciferase to β-galactosidase activity. The experiment was performed in triplicate and repeated at least twice; a representative experiment is shown.

RESULTS

Fatty acid regulation of COX-2 expression

Because prostaglandins are involved in many normal and pathophysiological responses, including tumor development, there is a need to understand how COX enzymes are regulated. Consideration of the possible pathways leading to increased COX-2 expression led to the question of whether fatty acids or their products could have an effect on either COX-1 or COX-2 expression. The first approach was to treat keratinocyte cultures with AA or its metabolites. Although keratinocytes in vivo do not express COX-2 (4), in culture untreated cells do express COX-2, presumably due to growth factors in the media. The addition of 10 μm AA was found to be sufficient to further induce COX-2 mRNA by 3.7-fold (this experiment) as compared to the solvent control by 30 min (**Fig. 1A**) and the effect was sustained for as long as 6 h after addition. Surprisingly, COX-1 was also induced 3-fold at 30 min and up to 5.6-fold by 90 min, although by 6 h COX-1 levels had dropped to below control levels. Repeated experiments (n = 9) demonstrated that at 3 h, 10 μm AA induced COX-2 by an average of 2.7-fold (±0.66 SD; $P < 0.001$) and induced COX-1 by 2.4-fold (±1.3SD; $P < 0.05$). COX-2 can also be induced by all three of the major prostaglandin metabolites of AA (Fig. 1B). By 3 h, PGD_2 (10 μm) is the most effective inducer, causing a 1.5-fold increase in COX-2 expression in this experiment; repeated experiments (n = 6; data not shown) produced an average 2.2-fold (±0.44 SD; $P < 0.01$) increase in mRNA levels. Like AA, PGD_2 induces COX-1 by a little over 2-fold (mean 2.3 ± 0.47; $P = 0.05$). PGE_2 was also effective in enhancing COX-2 levels (2.1 ± 0.80; n = 10; $P < 0.01$) while $PGF_{2\alpha}$ was a little less effective (1.8 ± 0.33; n = 4; $P < 0.1$); both induced COX-1 levels by approximately 2-fold (n = 7; $P < 0.01$ and n = 3; $P = 0.2$, respectively). Our observations of prostaglandin-induced COX-2 expression are in agreement with previous reports of autoregulation of COX-2 by prostaglandins in osteoblastic MC3T3-E1 cells (15, 16); however, we are not aware of previous reports showing that either AA or prostaglandins can induce COX-1.

We compared the effects of the polyunsaturated fatty acid (PUFA) linoleic acid (18:2 n–6, LA) and the saturated fatty acid stearic acid (18:0, SA) to AA on COX-1 and COX-2 expression levels in keratinocyte cultures, in part to determine whether the effect of AA on COX induction is an AA-specific or a general fatty acid effect. The cultures were treated for 3 h with 10 μm doses of LA, AA, or SA (Fig. 1B). All of the polyunsaturated fatty acids induced both COX-1 and COX-2 mRNA. The greatest induction of

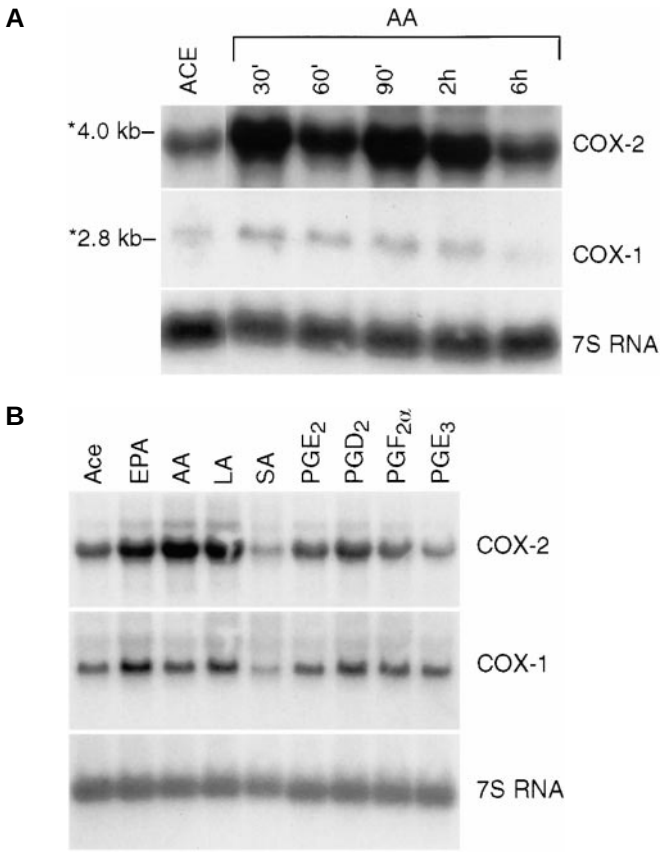

Fig. 1. Induction of COX-2 mRNA by arachidonic acid and various eicosanoids. Cultured murine keratinocytes were serum-starved for 48 h and then treated with (panel A) acetone (ACE) or 10 μm arachidonic acid (AA) for 0.5, 1, 1.5, 2, or 6 h or (panel B) acetone and 10 μm AA, eicosapentaenoic acid (EPA), linoleic acid (LA), stearic acid (SA), PGE_2, PGD_2, $PGF_{2\alpha}$ for 3 h. Total cellular RNA was isolated and examined by Northern analysis. Each lane contained 10 μg RNA. The blot was hybridized to ^{32}P-labeled COX-2 or COX-1 cDNAs and exposed to Kodak XAR-5 film with an intensifying screen overnight (representative experiment). A control for loading was obtained by stripping the blots of the COX-2 or COX-1 probes and rehybridizing with ^{32}P-labeled 7S RNA cDNA.

COX-2 mRNA was by AA (3-fold in this experiment) although EPA and LA were able to increase COX-2 expression by 2.2 (±0.24; n = 3; $P < 0.05$) and 2.5 (±0.4; n = 3; $P < 0.05$), respectively. COX-1 was also induced to a similar extent by all three polyunsaturated fatty acids. SA had little effect on either COX-2 or COX-1 mRNA levels suggesting that the fatty acid effects are not likely to be due to unspecific membrane perturbation events. It thus appears that there may be a relationship between the ability of a given fatty acid to induce both COX isoforms and the extent to which it can serve as a substrate for metabolizing enzymes.

To determine whether it is the prostaglandin products of AA that are primarily responsible for the induction of COX-1 or COX-2 mRNA in our keratinocyte cultures, we pretreated the cultures with 10 μm indomethacin, a COX-1 and -2 inhibitor (24) or NDGA (a lipoxygenase inhibitor) 30 min prior to stimulation with 10 μm AA (**Fig. 2**). AA-induced COX-2 mRNA was reduced to below basal levels by indomethacin pretreatment whereas NDGA was less effective. Indomethacin also reduced TPA-induced COX-2 expression by 30% (two experiments) although NDGA enhanced the effect of TPA for unknown reasons. Similar effects were seen for these drugs on AA or TPA-induced COX-1, i.e., indomethacin reduced COX-1 expression to 30% of the control level while NDGA reduced expression to the control level.

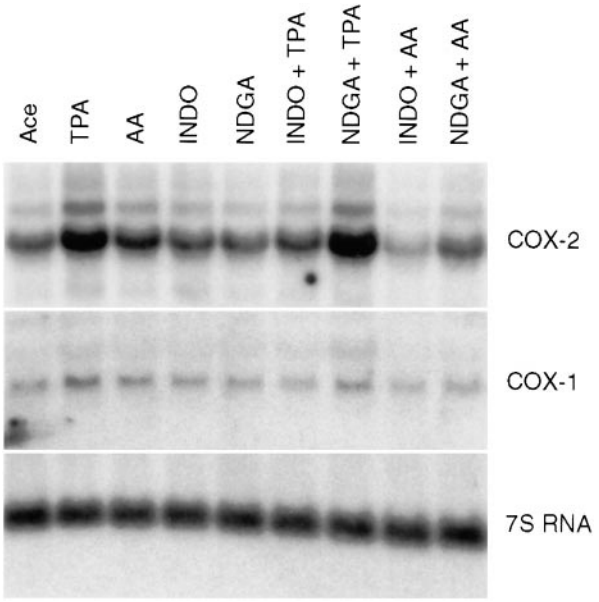

Fig. 2. Prostaglandins are partially responsible for the induction of COX-2 mRNA by arachidonic acid and 4β-12-O-tetradecanoylphorbol-13-acetate (TPA). Cultured murine keratinocytes were serum-starved for 48 h and then treated with acetone (Ace), TPA (1.6 μm), and 10 μm arachidonic acid (AA) in the presence or absence of 10 μm indomethacin (INDO) or nordihydroguaiaretic acid (NDGA) for 3 h. Total cellular RNA was isolated and examined by Northern analysis. Each lane contained 10 μg RNA. The blot was hybridized to ^{32}P-labeled COX-2 or COX-1 cDNAs and exposed to Kodak XAR-5 film with an intensifying screen overnight (representative experiment). A control for loading was obtained by stripping the blots of the COX-2 or COX-1 probes and rehybridizing with ^{32}P-labeled 7S RNA cDNA.

NS 398 inhibits AA-induced PGE_2 synthesis and COX-2 expression

Treatment of [^{14}C-]arachidonic acid pre-labeled murine keratinocytes with 10 μm cold AA resulted in AA release and subsequent PG synthesis. PGE_2 is the major PG synthesized by murine keratinocytes and was thus used as a marker of COX activity (25). To determine whether the subsequent metabolism to PGE_2 is mediated by COX-1 or COX-2, we used the COX-2 specific inhibitor NS 398 (26). PGE_2 synthesis was inhibited 73% with 1 μm NS 398 and 81% by 10 μm NS 398 (**Fig. 3**). We also examined the effect of NS 398 on COX-1 and COX-2 mRNA induced by AA. A 1 μm dose of NS 398 dramatically reduced both AA-induced COX-1 and COX-2 expression by 50 to 90% (duplicate experiments) (**Fig. 4**). These results suggest that AA-induced PGE_2 synthesis may contribute to the induction of both COX isoforms and is mediated in part by COX-2.

cAMP-mediated induction of COX-2 by AA

Treatment of murine keratinocytes with 10 μm forskolin, a known adenylate cyclase activator (**Fig. 5, left panel**) elevated both COX-1 and COX-2 expression by almost 2-fold (n = 3) by 30 min and this was sustained through 3 h. Treatment with 100 μm dibutyryl cAMP (Fig. 5, right panel), a cAMP analog, also induced both COX isoforms. Maximum induction occurred between 60 and 90 min with a 1.5- and 2.3-fold increase in COX-1 and COX-2, respectively. This suggested that a cAMP / PKA-mediated pathway may be functional in our keratinocyte cultures. To further investigate the involvement of a cAMP pathway in AA-induced COX-2 expression, we utilized the adenylate cyclase-

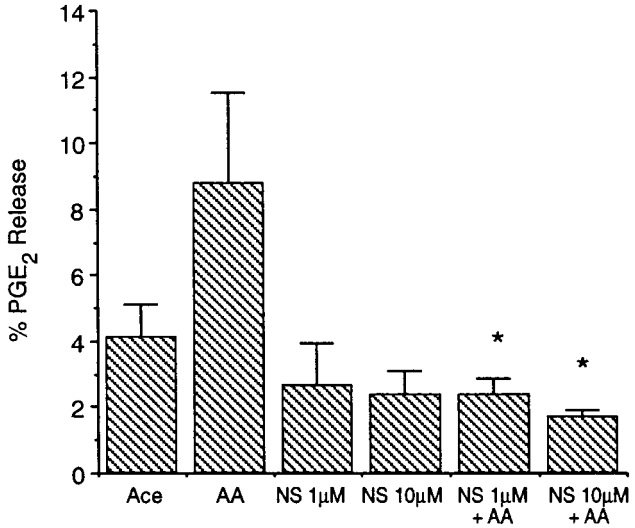

Fig. 3. Inhibition of PGE_2 synthesis by NS 398. Murine keratinocytes were labeled for 16 h with [^{14}C] arachidonic acid. After labeling, the media were removed and the dishes were re-fed with unlabeled conditioned media containing acetone (ACE) or 10 μm arachidonic acid (AA) in the presence or absence of NS 398 (1 or 10 μm) for 3 h. NS 398 was added 30 min prior to stimulation with arachidonic acid. After 3 h, 1-ml aliquots were taken and subjected to thin-layer chromatography to determine the amount of PGE_2 released. Bars represent the mean of 3 experiments ± standard deviation. * Statistically significant from AA ($P < 0.05$; ANOVA).

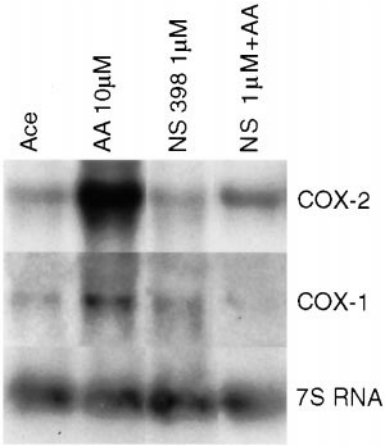

Fig. 4. Induction of COX-2 mRNA by arachidonic acid is inhibited by NS 398. Cultured murine keratinocytes were serum-starved for 48 h and then were treated with acetone (Ace) or 10 μm AA in the presence or absence of 1 μm NS 398 for 3 h. NS 398 was added 30 min prior to stimulation by AA. Total cellular RNA was isolated and examined by Northern analysis. Each lane contained 10 μg RNA. The blot was hybridized to ^{32}P-labeled COX-2 or COX-1 cDNAs and exposed to Kodak XAR-5 film with an intensifying screen overnight (representative experiment). A control for loading was obtained by stripping the blots of the COX-2 or COX-1 probes and rehybridizing with ^{32}P-labeled 7S RNA cDNA.

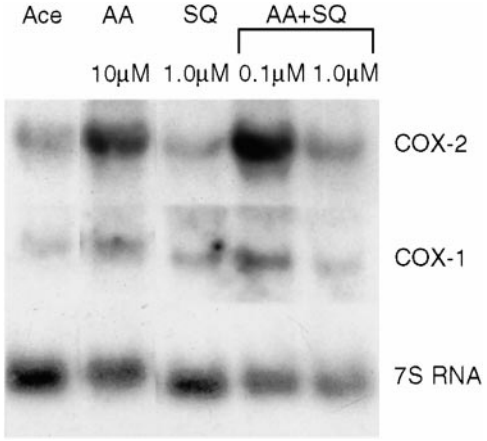

Fig. 6. Induction of COX-2 mRNA by arachidonic acid is inhibited by SQ 22536. Cultured murine keratinocytes were serum-starved for 48 h and then treated with acetone (Ace) or 10 μm arachidonic acid (AA) for 3 h in the presence or absence of the adenylate cyclase inhibitor SQ 22536. Inhibitor was added 30 min prior to stimulation with PGE_2. Total cellular RNA was isolated and examined by Northern analysis. Each lane contained 10 μg RNA. The blot was hybridized to ^{32}P-labeled COX-2 or COX-1 cDNAs and exposed to Kodak XAR-5 film with an intensifying screen (representative experiment). A control for loading was obtained by stripping the blots of the COX-2 or COX-1 probes and rehybridizing with ^{32}P-labeled 7S RNA cDNA.

specific inhibitor, SQ 22536. A 70% and 50% reduction (n = 2) in AA-induced COX-1 and COX-2 expression, respectively, was noted with a 1.0 μm dose of the adenylate cyclase inhibitor (**Fig. 6**) suggesting that either AA or prostaglandins may activate a cAMP / PKA signaling pathway.

cAMP-mediated induction of COX-2 expression by PGE_2

Although COX-2 expression can be induced by both AA and prostaglandins, we were particularly interested in identifying the mechanism(s) of the autoregulatory effect of PGE_2, the major prostaglandin synthesized by keratinocytes (5), on COX-2 induction. PGE_2 binds to and activates four receptor subtypes (EP1, 2, 3, and 4), three of which are linked to a cAMP signal transduction system (27). The fourth, the EP1 receptor, mediates smooth muscle contraction and is linked to extracellular Ca^{2+} influx (28). We ruled out the involvement of the EP1 receptor signal transduction pathway with use of AH 6809, an in-

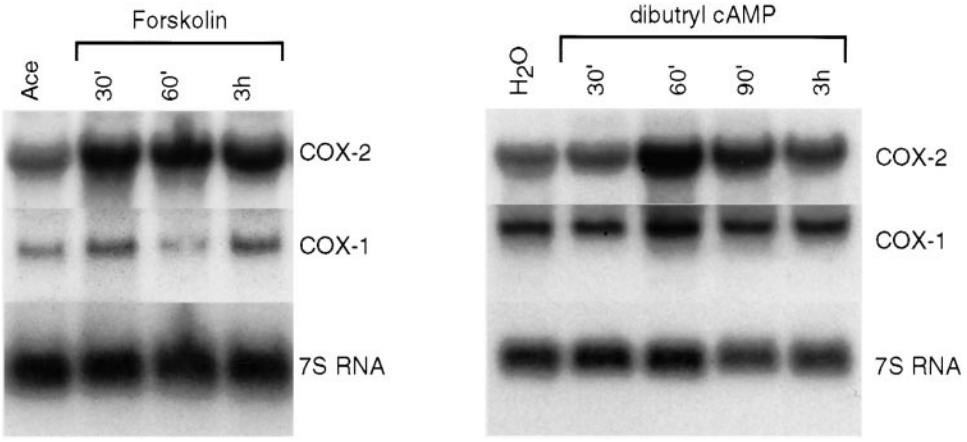

Fig. 5. Induction of COX-2 mRNA by activators of cAMP / PKA signal transduction pathway. Cultured murine keratinocytes were serum-starved for 48 h and then treated with acetone (Ace) or 10 μm forskolin for 0.5, 1, or 3 h (left panel) or solvent (H_2O; 10 μl / ml) or 100 μm dibutryl cAMP for 0.5, 1, 1.5, or 3 h. Total cellular RNA was isolated and examined by Northern analysis. Each lane contained 10 μg RNA. The blot was hybridized to ^{32}P-labeled COX-2 or COX-1 cDNAs and exposed to Kodak XAR-5 film with an intensifying screen (representative experiment). A control for loading was obtained by stripping the blots of the COX-2 or COX-1 probes and rehybridizing with ^{32}P-labeled 7S RNA cDNA.

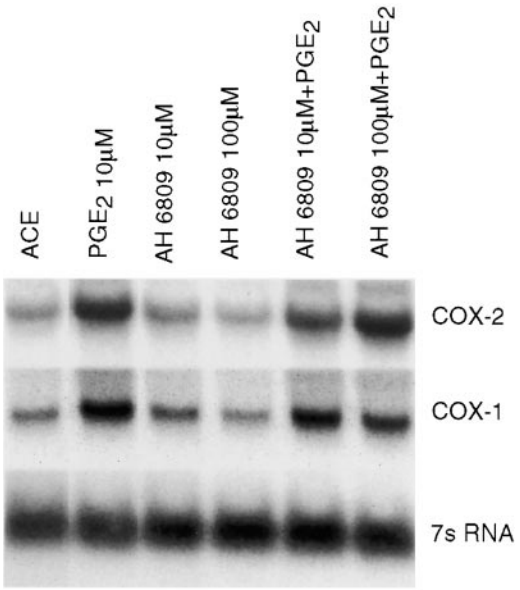

Fig. 7. Induction of COX-2 by PGE_2 is not inhibited by the EP1 receptor antagonist, AH 6809. Murine keratinocyte cultures were treated with 10 μm PGE_2 in the presence or absence of 10 or 100 μm AH 6809 for 3 h. The inhibitor was added 30 min prior to stimulation with PGE_2. Total cellular RNA was isolated and examined by Northern analysis. Each lane contained 10 μg RNA. The blot was hybridized to ^{32}P-labeled COX-2 or COX-1 cDNAs and exposed to Kodak XAR-5 film with an intensifying screen overnight (representative experiment). A control for loading was obtained by stripping the blots of the COX-2 or COX-1 probes and rehybridizing with ^{32}P-labeled 7S RNA cDNA.

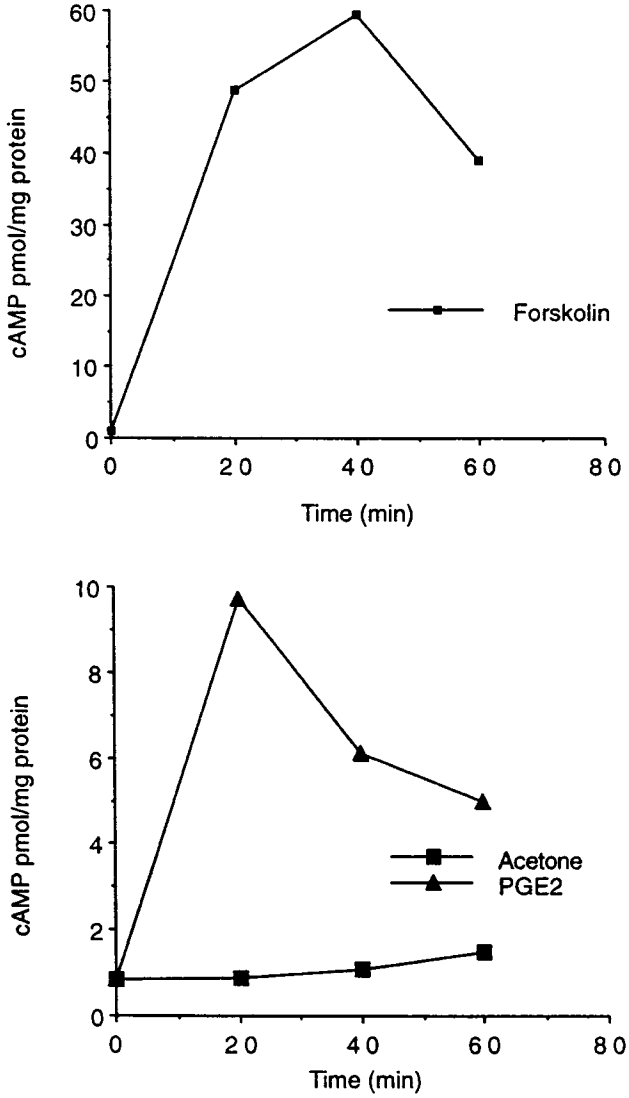

Fig. 8. cAMP is elevated by PGE_2. Murine keratinocytes were serum-starved for 48 h and then treated with 10 μm forskolin (top panel) or acetone (Ace) or 10 μm PGE_2 (bottom panel) for 3 h. The cAMP levels were determined by enzyme immunoassay from the supernatant of trichloroacetic acid-precipitated cells. The level of cAMP is expressed as pmol/mg protein. The data are representative of three separate experiments.

hibitor selective for the EP1 receptor subtype. At doses of 10 and 100 μm, we saw no inhibition (duplicate experiments) of the induction of either COX-1 or COX-2 expression by PGE_2, suggesting that this response is controlled by PGE_2 receptors linked to a cAMP signaling pathway (**Fig. 7**).

We looked at PGE_2 induction of cAMP in cultured murine keratinocytes and saw that 10 μm PGE_2 induced cAMP 9-fold 20 min after treatment as measured by cAMP enzyme immunoassay (**Fig. 8**). Forskolin (10 μm) was used as a positive control for our keratinocyte system and was an effective inducer of cAMP.

To further investigate the involvement of a cAMP-linked pathway in the PGE_2 induction of COX-2, we tested the effect of the adenylate cyclase inhibitor SQ22536 on COX-2 induction by PGE_2. Cultures were treated with increasing doses of SQ 22536 30 min prior to the addition of PGE_2, which dose-dependently reduced COX-2 expression. The induction of COX-2 expression by PGE2 was reduced by 50% (n = 2) by 10 μm SQ 22536 (**Fig. 9**). Interestingly, COX-1 levels, which were induced by PGE_2, were unaffected by SQ 22536. The reason for the lack of coordinate changes between COX-1 and COX-2 in this case is unknown.

Transcriptional activation of COX-2 by PGE_2

To determine whether the enhanced expression of COX-2 by PGE_2 was due to an increase in transcription or to stabilization of the message, we transiently transfected our keratinocytes with the full length COX-2 promoter linked to a luciferase reporter gene (**Fig. 10**), as we have previously reported (29). The luciferase reporter vector (pTIS10L) contains the promoter region of the mouse COX-2 gene −963/+70 from the transcription initiation site. Twenty-four hours after transfection, cultures were treated with 10 μm AA, PGE_2, PGD_2, or $PGF_{2\alpha}$ for 9 h. There was no effect on luciferase activity in the vector-only transfected cultures, but we saw an 11-fold increase in luciferase activity in the cultures transfected with the full-length promoter and treated with PGE_2. This suggests that PGE_2 can transcriptionally activate COX-2. Whether this is true for COX-1 remains to be determined.

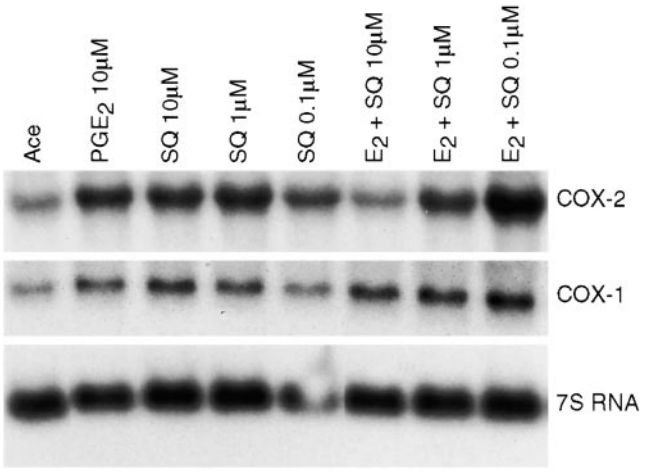

Fig. 9. Induction of COX-2 by PGE_2 can be inhibited by the adenylate cyclase inhibitor, SQ 22536. Cultured murine keratinocytes were serum-starved for 48 h and then treated with acetone (Ace), dimethyl sulfoxide (DMSO), 10 μm PGE_2 in the presence or absence of 0.1, 1.0, or 10 μm SQ 22536 for 3 h. The inhibitor was added 30 min prior to stimulation with PGE_2. Total cellular RNA was isolated and examined by Northern analysis. Each lane contained 10 μg RNA. The blot was hybridized to ^{32}P-labeled COX-2 or COX-1 cDNAs and exposed to Kodak XAR-5 film with an intensifying screen overnight (representative experiment). A control for loading was obtained by stripping the blots of the COX-2 or COX-1 probes and rehybridizing with ^{32}P-labeled 7S RNA cDNA.

DISCUSSION

It has been suggested previously that prostaglandins could autoregulate their synthesis (14–18). In our keratinocyte system we have demonstrated autoregulation of COX-1 and COX-2 by AA and the major prostaglandins that is mediated, in part, by cAMP. These events may partially contribute to the over-expression of prostaglandins and COX-2 observed in chronic inflammatory diseases and in skin and colon tumors from both humans and experimental animals (5).

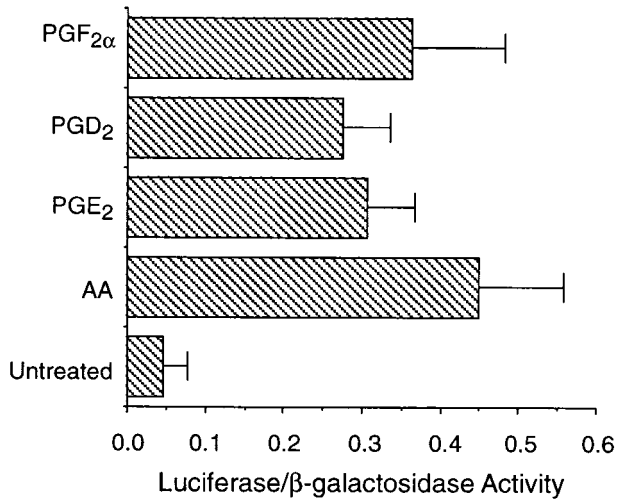

Fig. 10. COX-2 is transcriptionally activated by PGE_2. Keratinocytes were transfected with the PXP2 expression vector or the luciferase reporter vector containing the full-length COX-2 promoter and pRSVβ-gal using lipofectin. Twenty-four h after transfection, the cultures were treated with 10 μm arachidonic acid (AA), PGE_2 PGD_2, $PGF_{2\alpha}$ for 9 h. After treatment, cells were harvested and assayed for luciferase and β-galactosidase activity. Values are expressed as the mean ± standard deviation of the ratio of luciferase to β-galactosidase activity.

The role of PGE_2 in keratinocyte function is not entirely clear. While prostaglandins have not been shown to be involved in normal murine keratinocyte proliferation in vivo, PGE_2 was found to be a required co-mitogen for phorbol ester-elicited hyperproliferation and for mechanical wounding (30). In vitro, however, several studies suggested an involvement of PGs even in unstimulated proliferation. In human keratinocytes, a correlation between endogenous PGE_2 production and DNA synthesis was observed. Inhibition of proliferation by indomethacin can be overcome by exogenous addition of PGE_2, strongly suggesting that PGE_2 is a growth-promoting autocoid for the epidermis (31). Experimental models of skin cancer in mice have shown that COX inhibitors are effective in reducing tumor multiplicities and incidence (4, 7). However, the strongest support for a role for prostaglandins in skin tumor promotion comes from initiation–promotion studies with COX-1 and COX-2-deficient mice, in which a 70–80% reduction in papilloma number, compared with wild-type, was observed (32, 33).

As the actions of prostaglandins are mediated via cell surface receptors (9, 27), we evaluated the involvement of specific signaling pathways coupled to prostaglandin receptors in the induction of COX-1 and COX-2 by prostaglandins. We focused on PGE_2 inasmuch as affinity binding studies have identified receptors for this prostaglandin in primary mouse keratinocytes although the specific PGE_2 receptors present on murine keratinocytes have yet to be identified (34). Four types of PGE_2 receptors have been identified in other tissues that are coupled to either extracellular calcium influx or cAMP-mediated signal transduction pathways (35). EP1 receptors have been shown to mediate neurotransmitter release and smooth muscle contraction via extracellular calcium influx (28). EP2 receptors have been identified in epithelial cells and are linked to an increase in intracellular cAMP levels, whereas EP3 is also detected in epithelial cells but leads to a decrease in intracellular cAMP (9, 27). Recently, the existence of an EP4 subtype was identified in venous tissue and is coupled to stimulation of adenylate cyclase (33). In addition, three isoforms of the EP3 subtype have been identified and cloned (36, 37). While changes in calcium concentrations have been linked to increased COX-2 expression (38, 39), we saw no effect on PGE_2-induced expression of COX-2 when we used the EP1 antagonist AH 6809, suggesting that this receptor does not mediate the effects of PGE_2 in our keratinocytes. On the other hand, we demonstrated with the adenylate cyclase inhibitor, SQ 22536, that the action of PGE_2 on COX-2 expression is likely mediated, at least partially, by a cAMP pathway, possibly through EP2 or EP4 receptors. This is consistent with the report that adenylate cyclase-coupled EP receptors (EP2, 3 and 4) are expressed in human keratinocytes (40). The commercial

availability of specific PGE_2 receptor agonists and antagonists should help in the future identification of the particular receptor(s) responsible for the effect.

Unlike COX-2, COX-1 is expressed constitutively in most tissues except during development. COX-1 expression has also been shown to increase in two promonocytic cell lines, THP1 and U937, when induced to differentiate into macrophages (2). Stem cell factor and dexamethasone selectively induce COX-1 in mast cells (41). In both situations the expression is a characteristic of the differentiated cell. In light of these studies, the observation that eicosanoids can induce COX-1 in keratinocytes is intriguing, particularly in light of our observations that UV irradiation of murine skin can induce COX-1 as well as COX-2 and many UV-elicited skin tumors show constitutively elevated COX-1 expression (4). Thus the up-regulation of both COX-1 and COX-2 is significant and has implications in pathophysiological processes.

It has been known for some time that fatty acids can affect the expression of several genes, but the identity of a receptor protein(s) that could explain their pleiotropic responses has remained elusive. Recently, several target molecules have been shown to be activated by fatty acids or their metabolites (10–13). The identification of specific eicosanoids as natural ligands for peroxisomal proliferator-activated receptors (PPARs), a member of the nuclear steroid receptor superfamily, has raised speculation of a PPAR/fatty acid signal transduction mechanism (13, 42). Upon ligand binding, PPARs heterodimerize with the nuclear retinoic acid X receptor and bind to a peroxisome proliferator response element (PPRE) consensus sequence found in the enhancer region of target genes (43–45). Three PPAR subtypes with distinct tissue distribution have been identified in mice and are known to regulate a variety of genes involved in lipid metabolism (for review, ref. 46). We have observed increases in COX-2 mRNA by the peroxisome proliferator WY-14,643 and the PPARγ activator 15-delta-PGJ_2 (data not shown) similar to that reported by others in human mammary epithelial cells (47). In this latter study a PPAR response element was identified in the COX-2 promoter (47). As all three PPAR subtypes have been identified in keratinocytes (data not shown) the data collectively suggest that a PPAR pathway may also contribute to the autoactivation of COX-2 by prostaglandins as well as fatty acids.

In summary, our data suggest that *i*) COX-1 can be up-regulated by many of the same factors that up-regulate COX-2; *ii*) the mechanism(s) of COX-1 up-regulation is likely to be the same as for COX-2 as the two COX isoforms appear to be coordinately regulated in many circumstances; *iii*) COX-1 and COX-2 expression can be induced by arachidonic acid and its COX products, the prostaglandins; and *iv*) PGE_2-induced COX expression appears to be mediated by increases in cyclic nucleotides which occur as a result of binding and activation of specific cell surface receptors. The observed up-regulation of COX-1 is potentially important in many pathological processes in skin and may have bearing on the use of selective COX-2 inhibitors (4). Additional work is needed to clarify this issue.

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