Mechanism of 12-O-Tetradecanoylphorbol-13-acetate Enhanced Metabolism of Arachidonic Acid in Dog Urothelial Cells

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ABSTRACT

The mechanism of 12-O-tetradecanoylphorbol-13-acetate (TPA) enhanced arachidonic acid metabolism was investigated in dog urothelial cells. Primary cultures of dog urothelial cells were grown to confluency and evaluated in the presence or absence of overnight prelabeling with [3H]arachidonic acid. High-performance liquid chromatography analysis of media from TPA stimulated cells indicated that prostaglandin E2 (PGE2) was the major eicosanoid produced. Lipoxygenase products were not detected. Control cell media contained only arachidonic acid. Effects of selected inhibitors on TPA and exogenous arachidonic acid mediated increases in radioimmunoassayable PGE2 were assessed. Prostaglandin H synthase inhibitors (indomethacin and aspirin) prevented both TPA and arachidonic acid increases in PGE2. By contrast, inhibitors of phospholipases (quinoxine, W-7, and trifluoropromazine), protein synthesis (cycloheximide), and protein kinase C (staurosporine) prevented TPA but not arachidonic acid increases in PGE2. The latter agents also reduced TPA mediated increases in the release of total radioactivity from cells labeled with [3H]arachidonic acid. However, aspirin reduced the amount of 3H-prostaglandins formed with TPA. A calcium requirement was demonstrated when increases in radioimmunoassayable PGE2 eluted by TPA and the calcium ionophore A23187 were reduced with calcium depleted media. When epidermal growth factor in combination with either TPA or bradykinin was used, at least additive effects were observed with respect to release of [3H]arachidonic acid, [3H]-prostaglandins, and radioimmunoassayable PGE2. These experiments suggest that separate pathways may be involved in enhanced arachidonic acid metabolism demonstrated with different agonists. For TPA, increased arachidonic acid release occurs by a calcium dependent process involving phospholipase(s), protein synthesis, and protein kinase C.

INTRODUCTION

Hormones and growth factors are required to maintain cell growth, proliferation, and differentiation. They communicate their information across cell membranes by second messenger systems (signal transduction). Second messengers include cyclic AMP, inositol 1,4,5-triphosphate, and diacylglycerol (1-3). Eicosanoids produced by arachidonic acid metabolism can serve as second or subsequent messengers in cells (4, 5). Eicosanoids are also involved in several steps in the carcinogenic process (i.e., initiation, promotion, differentiation, and metastasis) (6-9). TPA: tumor promoter TPA mimics the actions of the second messenger diacylglycerol on protein kinase C (10). Tumor promoting effects of TPA in the mouse skin model are mediated, at least in part, by its activation of protein kinase C (11) and involve arachidonic acid metabolism (12).

of signal transduction pathways in carcinogenesis is further emphasized by the homology of several oncogenes to various components in these pathways (13) and the demonstration that ectopic expression of receptors can cause malignant transformation (14). Thus, an understanding of molecular mechanisms related to transmembrane signaling and second messenger systems will provide additional information about the multistep carcinogenic process.

Dog is the animal model for studying aromatic amine induced bladder cancer (15, 16). Dog urothelial cells metabolize aromatic amine bladder carcinogens (17, 18) and undergo unscheduled DNA synthesis in response to these carcinogens (19, 20). Bladder malignancies are carcinomas derived from urothelial cells. Interest in signal transduction pathways has resulted in the initiation of studies evaluating regulation of arachidonic acid metabolism in urothelial cells. Arachidonic acid metabolism was evaluated in dog relative to human urothelial cells. Results indicate that similar cellular regulatory processes exist in each species (21-24). For example, the same agonists were shown to increase PGE2 production in both species with subculturing and the absence of serum or the high concentration of serum reducing responsiveness. Thus, urothelial cell signal transduction pathways can be evaluated by examining regulation of arachidonic acid metabolism. In the present study, the profile of eicosanoids produced from control and TPA stimulated cells was determined. In addition, the mechanism of TPA enhanced arachidonic acid metabolism was detailed and compared to that of other agonists. These agonists include the polypeptide hormones bradykinin and epidermal growth factor and the calcium ionophore A23187 (24). This diverse group of agonists was chosen to maximize the information obtained. Results indicate similarities and differences in the mechanisms by which agonists influence cell signal transduction pathways in dog urothelial cells.

MATERIALS AND METHODS

Preparation of Cell Cultures. Urothelial cells from the urinary bladder of the dog were prepared and cultured by the modification of a method used for human urothelial cells (21, 25) as previously described (24). Using aseptic procedures, freshly detached cells were centrifuged at 1500 rpm for 10 min at 4°C. The cells in the pellet were carefully resuspended in a small volume of medium. Approximately 5 x 10^5 freshly detached cells in 3 ml of medium containing 1% fetal calf serum were added to 35-mm plastic tissue culture plates coated with 0.2% gelatin (Fisher Scientific Co., St. Louis, MO) and incubated at 37°C in 5% CO2-95% humidified air with medium changed biweekly. Medium consisted of Ham's nutrient mixture F12 (GIBCO, Grand Island, NY) supplemented as previously described (25).

Experiments were conducted with 80-90% confluent cultures of dog urothelial cells (3-4 x 10^5 cells/35 mm plate). Growth media were aspirated, and cells were washed twice with 1 ml of Hank's balanced salt solution. Serum-free α-MEM was then added with or without test agents as indicated in the “Results.” Cells were incubated at 37°C in 5% CO2-95% air. Test agents used were arachidonic acid, purchased from Nu-Chek Prep, Inc., Elysian, MN; calcium ionophore A23187 and staurosporine from Cal-

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biochem, La Jolla, CA; and cycloheximide, quinacrine, W-7, trifluoromizone, indomethacin, aspirin, bradykinin triacetate, epidural growth factor, and TPA from Sigma Chemical Co., St. Louis, MO. Each agonist was used at a maximally effective concentration, previously determined (24).

Prelabeling. Urothelial cells were rinsed twice with serum-free media and incubated with 1 μCi [3H]arachidonic acid (100 Ci/ml; Du Pont NEN Research Products, Boston, MA). After 1 h, fetal calf serum was added to a final concentration of 1%, and cells were incubated overnight (24). Cells were then rinsed twice with a-MEM containing 5 mg/ml BSA and twice with a-MEM containing 1 mg/ml BSA. All subsequent conditions contained a-MEM with 1 mg/ml BSA. After a 30-min preincubation in the presence or absence of inhibitors, agonists were added in fresh media containing the corresponding inhibitor. Media were analyzed for products of arachidonic acid metabolism.

Analysis of Arachidonic Acid Metabolites in Media. Arachidonic acid metabolites were identified by HPLC analysis. Media were acidified to pH 3.5 with glacial acetic acid, ethanol was added to a final concentration of 10%, and media were applied to a disposable extraction (PrepSep-C18) column. Retained material was eluted with methanol, concentrated, and used for subsequent analysis. Metabolites were separated by reverse-phase HPLC, with a C18 Utrasphere ODS column (Altex Scientific, Inc., Beckman Instruments, Inc., Berkeley, CA) using methanol and buffered water (pH 5.05) at a flow rate of 1.1 ml/min as described previously (26). The PGE2 and PGD2 fraction eluting from the reverse-phase HPLC column was collected, concentrated, and reappied to a straight-phase column (μ-Porasil, 10 μm, from Waters Associates, Milford, MA). The prostaglandins were eluted with hexane:ethanol:acetic acid (99:4:6:1) for 25 min followed by a linear gradient to 80% solvent of hexane:ethanol:acetic acid (90:10:1) for an additional 60 min at a flow rate of 3.0 ml/min (27).

To determine effects of inhibitors on radiolabeled nonpolar versus polar material in media, solvent extraction was used as previously described (28). The percentage of arachidonic acid (nonpolar) and prostaglandins (polar) was determined. Protein was precipitated by centrifugation following addition of two volumes of cold acetone. Media were then extracted with two volumes of petroleum ether. After removal of the organic phase, the aqueous fraction was extracted another two times with petroleum ether. All organic phases were combined and evaporated. Radioactivity in the organic and aqueous fractions was expressed as percentage of arachidonic acid and prostaglandins, respectively. The organic and aqueous fractions were characterized by thin-layer chromatographic analysis using silica gel F254-254. Plates were developed with chloroform:methanol:acetic acid (90:5:5). Prostaglandin standards and arachidonic acid were applied as carriers and visualized with iodine vapors. The radioactivity of zones corresponding to standards was determined (29). The organic fraction was found to contain arachidonic acid, whereas the aqueous fraction was predominantly prostaglandins. PGE2 was the major prostaglandin. These results are qualitatively consistent with those obtained with HPLC analysis and reported in Fig. 1.

Radioimmunoassay of Prostaglandin E2. The PGE2 content of the media was measured by double-antibody radioimmunoassay (30). Rabbit antiserum to PGE2 was obtained from Regis Chemical Co., Morton Grove, IL. Tritiated-labeled PGE2 was purchased from Du Pont NEN Research Products, and goat antiserum to rabbit γ-globulins was purchased from Antibodies, Inc., Davis, CA. The medium from each plate was analyzed in duplicate, and the value of this duplicate determination was averaged and considered as N = 1. Data were expressed as the mean of duplicate experiments or as the mean ± SEM of ng PGE2/106 cells. All experiments were repeated at least three times.

RESULTS

Metabolite Characterization. The profile of eicosanoids produced by urothelial cells was assessed using [3H]arachidonic acid prelabeled cells. Media from control and cells stimulated with TPA for 2 h were assessed by reverse-phase HPLC. As shown in Fig. 1, arachidonic acid was the only radiolabeled compound detected in control media. Following TPA stimulation, additional peaks of radioactive eicosanoids were observed. The peak which coeluted with the PGE2 and PGD2 standards contained the most radioactivity (25% of recovered radioactivity). Two small peaks were observed that coeluted with 6-keto-PGF1a (6%) and 12-hydroxy-5,8,10-heptadecatrienoic acid (2.5%) but were not further characterized. Leukotrienes (i.e., LTB4) and hydroxyeicosatetraenoic acids (i.e., 15-HETE and 5-HETE) were not detected, indicating the lack of lipoxygenase activity.

The peak of radioactivity which elutes with the PGE2 and PGD2 standards in Fig. 1 (20–22 min) was further analyzed by straight-phase HPLC. A single peak was observed that coeluted with PGF1α standard and confirmed the identity of the major eicosanoid product as PGE2. Thus, in subsequent experiments, PGE2 was determined by radioimmunoassay. The amount of radioactivity present in media from cells treated with TPA was significantly higher than that from control cells, suggesting that TPA may act in part by stimulating phospholipase activity.

Effects of Inhibitors. To further characterize the response to TPA, specific inhibitors were used (Fig. 2). TPA increased PGE2 formation from endogenous arachidonic acid from 0.27 ± 0.09 in control (data not shown) to 3.52 ± 0.44 ng/106 cells as measured by radioimmunoassay. PGE2 synthesis was significantly decreased in the presence of inhibitors of prostaglandin H synthase (indomethacin), phospholipases (quinacrine, W7, and trifluoromizone), protein synthesis (cycloheximide), or protein kinase C (staurosporine). Indomethacin and quinacrine were the most effective and trifluoromizone the least effective inhibitors. The concentrations of inhibitors used were maximal, as determined in previous experiments. A lower concentration of cycloheximide (10 μM) than used in Fig. 2 inhibits protein synthesis in human urothelial cells by 90–95% (22).

Effects of these inhibitors on PGE2 production from exogenous arachidonic acid were investigated (Fig. 2). The use of exogenous arachidonic acid bypasses the requirement for phospholipase activity (endogenous arachidonic acid) and focuses specifically on the ability of the prostaglandin H synthase enzyme to produce PGE2. The addition of arachidonic acid to cells increased PGE2 from 0.27 ± 0.09 in control (data not shown) to 6.58 ± 0.87 ng/106 cells. Indomethacin and aspirin (data not shown) significantly reduced arachidonic acid increases in PGE2. Inhibitors of phospholipases, protein synthe-
STIMULATION OF PGE\textsubscript{2} PRODUCTION BY TPA

Fig. 2. Effects of different inhibitors on TPA and exogenous arachidonic acid increases in radioimmunoassayable PGE\textsubscript{2}. Cells were preincubated with indo-methacin (INDO), quinacrine (QUIN), W-7 (W7), trifluoropromazine (TFP), cycloheximide (CYCLO), or staurosporine (STAURO). PGE\textsubscript{2} was assessed 2 h after addition of TPA (100 nM) or arachidonic acid (100 \mu M). The amount of PGE\textsubscript{2} in control media was 0.27 ± 0.09 ng/10\(^6\) cells.

Fig. 3. Effects of different inhibitors on TPA mediated release of endogenous arachidonic acid. [\(^{3}\text{H}\)]Arachidonic acid prelabeled cells were preincubated with the same inhibitors used in Fig. 2. Media radioactivity was assessed 2 h after addition of 100 nM TPA. The amount of radioactivity in media from control cells was 19,600 ± 940 cpm.

Fig. 4. Effects of aspirin on release of [\(^{3}\text{H}\)]arachidonic acid, [\(^{3}\text{H}\)]prostaglandins, and radioimmunoassayable PGE\textsubscript{2} elicited by TPA. Cells were pretreated in the presence and absence of 1 mM aspirin (ASA) for 30 min and then TPA for 2 h. Both unlabeled and [\(^{3}\text{H}\)]arachidonic acid prelabeled cells were used. With the former, radioimmunoassayable (RIA) PGE\textsubscript{2} was assessed in media. Media from prelabeled cells were monitored for [\(^{3}\text{H}\)]arachidonic acid (AA) and [\(^{3}\text{H}\)]prostaglandins (PGs). See details in “Materials and Methods.”

Fig. 5. Calcium requirement for agonist stimulated PGE\textsubscript{2} synthesis was assessed. Cells were preincubated for 30 min in the presence or absence of calcium depleted media containing 0.05 mM EDTA. Agonists were added for 1 h, and media PGE\textsubscript{2} content was assessed. The absolute response was obtained by subtracting control from agonist stimulated values. Control values in the presence and absence of calcium were 5.8 ± 0.7 and 1.9 ± 0.5 ng/10\(^6\) cells, respectively.

Prostaglandin H synthase. Thus, prostaglandins synthesized following aspirin treatment would require synthesis of new enzyme. [\(^{3}\text{H}\)]Arachidonic acid prelabeled cells were pretreated with 1 mM aspirin for 30 min and then washed twice to remove aspirin before incubation with TPA (Fig. 4). The amount of radiolabeled prostaglandins was completely abolished. In a parallel experiment, TPA increases in radioimmunoassayable PGE\textsubscript{2} were correspondingly inhibited by preincubation of cells with aspirin. However, the amount of radiolabeled arachidonic acid released was not significantly altered by aspirin pretreatment. Similar effects were observed following bradykinin addition to aspirin treated cells (data not shown). Thus, these results suggest that the agonists TPA and bradykinin are not inducing the synthesis of prostaglandin H synthase.

Calcium Requirement. The calcium requirement for agonist stimulated PGE\textsubscript{2} production was evaluated (Fig. 5). Cells were preincubated in media that contained or was devoid of calcium following aspirin treatment would require synthesis of new enzyme.
Effect of Other Agonists. The effect of other agonists on arachidonic acid metabolism was evaluated (Fig. 6). EGF was evaluated alone and in combination with TPA and bradykinin. Each agonist was used at maximum effective concentrations (24). Radioactive and nonradioactive experiments were done in parallel. EGF produced modest increase in each parameter. Increases in radioimmunoassayable PGE2 were significantly greater in the presence than absence of EGF. In addition, the amount of 3H-prostaglandins observed was greater in the presence than absence of EGF. However, increases were only significant with bradykinin plus EGF compared to bradykinin alone. Values observed with release of [3H]arachidonic acid were increased in the presence compared to the absence of EGF, although none achieved significance.

DISCUSSION

This is the first investigation to determine the profile of eicosanoids produced by dog urothelial cells. Cellular prostaglandin H synthase was demonstrated by the detection of prostaglandins. PGE2 was the most abundant eicosanoid produced. 6-Keto-PGF1α, and HHT were also detected in smaller amounts and only tentatively identified. 6-Keto-PGF1α is a decomposition product of PGF1α metabolism (37). HHT is a product of both the thromboxane and prostaglandin pathways. However, because thromboxane was not detected, HHT was probably derived from the prostaglandin pathway (38). These results are similar to those of a previous study assessing arachidonic acid metabolism of human urothelial cells (21). In human cells, PGE2 is the major eicosanoid synthesized, with significant amounts of 6-keto-PGF1α and HHT also being detected. HPLC analysis of dog urothelial cell media failed to detect lipoxygenase products including leukotrienes. In contrast, human cells produced small (6% of the total eicosanoids detected) but significant amounts of lipoxygenase products. The latter were only tentatively identified. The current study does not rule out the possible presence of lipoxygenases in dog urothelial cells. Other unsaturated lipids may prove to be better substrates for these enzymes than arachidonic acid (39, 40). Thromboxane and leukotrienes were not detected with either dog or human urothelial cells.

The increase in prostaglandin production observed with TPA and other agonists has been attributed to de novo synthesis of the enzyme prostaglandin H synthase (31–34, 41–43). In those studies, cycloheximide prevented the increase in prostaglandin H synthase and the subsequent increase in prostaglandin production. Aspirin is an irreversible inhibitor of prostaglandin H synthase (36). Thus, new enzyme synthesis is required before eicosanoids can be produced. In cells pretreated with aspirin, agonist (i.e., TPA) increases in prostaglandin production due to synthesis of new enzyme are not prevented (31, 42, 43). In the current study, cycloheximide prevented increases in PGE2 elicited by TPA. However, aspirin pretreatment prevented TPA increases in PGE2 and radiolabeled prostaglandins but not the release of arachidonic acid. Thus, TPA does not appear to be increasing synthesis of new prostaglandin H synthase enzyme during the 2-h incubation period. TPA mediated increases in urothelial cell prostaglandin H synthase synthesis may occur later.

Increased availability of arachidonic acid substrate is another mechanism by which agonists regulate increases in prostaglandin production. TPA dramatically increased the release of radioactivity from [3H]arachidonic acid prelabeled urothelial cells. These results are consistent with this material being initially arachidonic acid. That is, arachidonic acid is the only compound identified in media from TPA stimulated cells pretreated with aspirin (Fig. 4). Thus, TPA elicits an increased release of endogenous arachidonic acid which is metabolized by prostaglandin H synthase to prostaglandins, primarily PGE2. Cycloheximide treatment reduced the TPA mediated release of radioactivity and synthesis of PGE2 which is consistent with reduced availability of arachidonic acid. Inhibitors of phospholipases (quinacrine, W-7, and TFP) (44) and protein kinase C (stauroporine) (45) also inhibited both release of radioactivity and PGE2 production mediated by TPA. The lack of effect of these inhibitors on increased PGE2 production mediated by exogenous arachidonic acid demonstrates the specificity of their effects (Fig. 2). Thus, TPA regulated increases in eicosanoids appear to require protein synthesis and involve phospholipase(s) and protein kinase C dependent pathways.

In this study, treatment of cells with cycloheximide prevented the increase in prostaglandin production and suggests that protein synthesis is required for TPA stimulation. The dependency of arachidonic acid release on protein synthesis and gene expression has been previously observed. Clark et al. (46) have shown that cycloheximide and actinomycin D prevented the leukotriene D4 stimulated release of arachidonic acid and eicosanoid production by smooth muscle and endothelial cells. The stimulatory effects of leukotriene D4 were attributed to synthesis of a phospholipase A2 stimulatory peptide (47). In human and dog urothelial cells, TPA increases in PGE2 production are time delayed and dependent upon protein synthesis (22, 24). The present study is the first to demonstrate that cycloheximide actually blocks urothelial cell release of radioactivity mediated by TPA. In dog urothelial cells, TPA increased PGE2 synthesis is also prevented by actinomycin D (24). Although our results indicate dependency of arachidonic acid release on protein synthesis and gene expression, it is not clear whether phospholipase A2 and/or phospholipase C pathways are involved.

Protein kinase C is the major cellular receptor for TPA (10). The tumor promoting effects of TPA in the mouse skin model are mediated, at least in part, through protein kinase C (11). Protein kinase C has been suggested to phosphorylate and modulate the activity of a family of proteins (lipocortins) that inhibit phospholipase A2 (48). Phosphorylation mediated activation of phospholipase A2 is thought to be responsible for TPA mediated increased release of arachidonic acid and prostaglandin synthesis in MDCK cells (49). Stauroporine inhibited...
tion of TPA mediated urothelial cell release of radioactivity and synthesis of PGE₂ suggests that protein kinase C is involved in regulation of arachidonic acid metabolism in this model system.

Calcium can function as a second messenger and/or be a necessary cofactor for subsequent steps in signal transduction pathways. Previous studies including those with human urothelial cells have demonstrated a calcium requirement for TPA responses (22). Phospholipase A₂ (50), phospholipase C (51, 52), and protein kinase C (10) require calcium. Calcium requirements of the latter enzymes could explain the reduced synthesis of PGE₂ observed in calcium-depleted media with TPA and A23187.

Effects of inhibitors have been attributed, in the preceding discussion, to specific processes. However, other effects of these agents should be considered. Trifluoperazine and W-7 are classified as calmodulin antagonists. Calmodulin antagonists including W-7 have antiproliferative and antitumor effects (53). Since calmodulin does not activate purified phospholipase A₂ (44), the effects of trifluoperazine and W-7 on arachidonic acid release may relate to the calcium dependency of this enzyme or involve phospholipase C. The proposed effects of trifluoperazine and W-7 on phospholipase(s) are consistent with the inhibition observed with quinacrine. Quinacrine is often used to assess the involvement of phospholipase pathways (44). High concentrations of nonsteroidal antiinflammatory agents may inhibit phospholipase A₂ (44). However, nonsteroidal antiinflammatory agents did not reduce agonist-mediated release of arachidonic acid. Instead, they prevented increases in PGE₂ production by way of their inhibitory effects on the fatty acid cyclooxygenase activity of prostaglandin H synthase. Although the inhibitors used have a variety of effects on different processes, the use of multiple inhibitors with different chemical structures that have overlapping effects on phospholipases suggests a role for these enzymes in TPA-mediated PGE₂ synthesis.

Cell viability was used as a basis for selecting maximally effective concentrations of test agents. Cell count and arachidonic acid responsiveness were used as indexes of cell viability. When conducting dose response studies, concentrations of test agents which decreased cell count or arachidonic acid stimulation of cell count or arachidonic acid stimula
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