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Requirement for Epidermal Growth Factor Receptor Tyrosine Kinase and for 12-Lipoxygenase Activity in the Expression of 12-Lipoxygenase in Human Epidermoid Carcinoma Cells

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ABSTRACT. We studied the dependency of basal 12-lipoxygenase (12-LOX; arachidonate:oxygen 12-oxidoreductase, EC 1.13.11.31) expression and activity on functional protein tyrosine kinase of the epidermal growth factor receptor (EGF-R) and on 12-LOX activity in human A431 epidermoid carcinoma cells. Treatment of cells with inhibitors of high specificity for EGF-R tyrosine kinase, namely PD 153035 and 4,5-dianilinophthalimide (DAPH1), decreased cellular 12-LOX at mRNA, protein, and activity levels in a time- and dose-dependent manner, with PD 153035 being effective at concentrations below 1 μM. After 24-hr incubation with 10 μM PD 153035 or DAPH1, 12-LOX activity dropped to 14% (39%), and 12-LOX protein to 25% (24%) of control level. Inhibition of 12-LOX activity by the compound *N*-benzyl-*N*-hydroxy-5-phenylpentanamide (BHPP) also resulted in a substantial decrease in 12-LOX protein expression. 12-LOX mRNA levels were diminished or undetectable by reverse transcription-polymerase chain reaction after cell treatment with these inhibitors. Our results suggest that basal 12-LOX expression in A431 tumor cells largely depends on functional EGF-R tyrosine kinase, and that 12-LOX activity is required in the EGF-elicited intracellular signaling maintaining the expression of 12-LOX. BIOCHEM PHARMACOL 53;7:937–942, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. epidermal growth factor; tyrosine kinase; 12-lipoxygenase; 12-lipoxygenase inhibitor; EGF-R tyrosine kinase inhibitor

The epidermal growth factor receptor (EGF-R†) mediates cellular signaling to epidermal growth factor (EGF) and to transforming growth factor α [1]. EGF-R participates in epithelial cell proliferation, and there is strong evidence suggesting that EGF-R plays a role in malignant tumor growth. Overexpression of EGF-R can result in a neoplastic phenotype in transgenic mice [2] and cells [3]. The EGF-R consists of a transmembrane glycoprotein, the intracellular domain of which exhibits protein tyrosine kinase activity [1]. This tyrosine kinase of the EGF-R is essentially involved in intracellular transmission of EGF signaling, which includes activation of various phospholipases such as phospholipase A_2 (PLA_2) [4], phospholipase C-γ (PLC-γ) [5], and phospholipase D (PLD) [6], and subsequently, activation of lipoxygenases (LOXs) [7–9]. At the 12-LOX (arachidonate:oxygen 12-oxidoreductase, EC 1.13.11.31) level, EGF has also been shown to induce both mRNA expression and enzymatic activity levels of 12-LOX in A431 cells within several hours [10, 11], with the latter due to an increased intracellular translocation to membraneous sites including nuclei [12]. We recently demonstrated that EGF can also elicit 12-LOX activity in the presence of sufficient intracellular free Ca^{2+} within minutes [12].

Our interest in 12-LOX is based on the pleiotropic prometastatic effects known to be elicited via 12-LOX action by the arachidonate metabolite 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) [13]. These actions of 12(S)-HETE include increase in tumor cell adhesion to vascular endothelium [14] and integrin expression [15], retraction of vascular endothelial cells [16], stimulation of tumor cell motility [17], and invasiveness [18] via activation of protein kinase C [18], as well as enhanced release of cathepsin B [19]. On the other hand, some tumor cells including human epidermoid carcinoma A431 cells and human erythroleukemia (HEL) cells express the platelet-type isoform of 12-LOX [11, 12, 20], and tumor cell 12-LOX has been shown

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† *Abbreviations:* BHPP, *N*-benzyl-*N*-hydroxy-5-phenylpentanamide; DAPH1, 4,5-dianilinophthalimide; EGF, epidermal growth factor; EGF-R, EGF-Receptor; HEL, human erythroleukemia; 12(S)-HETE, 12(S)-hydroxyeicosatetraenoic acid; 12(S)-HPETE, 12(S)-hydroperoxyeicosatetraenoic acid; IC_{50}, concentration needed for half-maximal inhibition; 12-LOX (5-LOX, 15-LOX), 12-lipoxygenase (5-lipoxygenase, 15-lipoxygenase); PLA_2 (PLC-γ, PLD), phospholipase A_2, (phospholipase C-γ, phospholipase D); PCR, polymerase chain reaction.

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to contribute to tumor cell metastatic potential *in vivo* and *in vitro* [21].

Cellular 12-LOX expression is not necessarily constitutive [13] and can be modulated in a bidirectional manner [12]. In view of the suggested role of tumor cell 12-LOX in the process of metastasis, we wondered whether inhibition of 12-LOX expression in tumor cells represented a suitable target for future intervention of their metastatic potential. Because general inhibition of protein tyrosine kinase is sufficient to suppress expression of 12-LOX in A431 cells [12], we investigated in this study the possible participation of the EGF-R tyrosine kinase on basal 12-LOX expression and activity. Recently, two compounds with high selectivity for inhibition of EGF-R tyrosine kinase and potent antitumor action have been reported, namely 4,5-dianilinophthalimide [DAPH1] [22] and PD 153035 [23]. Using these EGF-R tyrosine kinase inhibitors and the LOX inhibitor *N*-benzyl-*N*-hydroxy-5-phenylpentanamide (BHPP) [21], we demonstrate here with A431 tumor cells that: (a) inhibition of EGF-R tyrosine kinase results in substantial suppression of 12-LOX expression; (b) the inhibitors of EGF-R tyrosine kinase do not directly inhibit 12-LOX activity; and (c) functional 12-LOX activity is involved in maintaining expression of 12-LOX protein.

MATERIALS AND METHODS

Tumor Cells

The human epidermoid carcinoma A431 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured at 37°C and 5% (v/v) CO_2 in Dulbecco's modified Eagle's medium containing 4.5 g of glucose/L, 10% (v/v) fetal calf serum, streptomycin (100 μg/mL), and penicillin (100 units/mL). Viability of cells was tested via trypan blue exclusion and always amounted to >95%.

Reverse Transcription-Polymerase Chain Reaction (PCR)

Total RNA was obtained using guanidinium thiocyanate according to [24] and suspended at 1 μg/μL in water. Three micrograms of RNA were reverse-transcribed using oligo$(dT)_{18}$ primers, and 2 μL of the cDNA mixture were amplified by PCR with sense and antisense primers specific for platelet 12-LOX; both of these primers spanned exon-intron boundaries of the 12-LOX gene and thus avoided amplification of genomic DNA during PCR. After heating the PCR mixture for 3 min at 94°C, amplification was performed at 94°C, 30 sec; 70°C, 30 sec; 72°C, 30 sec for 35 cycles (12-LOX) or at 94°C, 1 min; 60°C, 1 min; 72°C, 2 min for 31 cycles (β-actin). The sequences of the 12-LOX primers used in this study were: sense, 5′-GCC AGG TAT GTG GAG GGG ATC-3′ (identical to nucleotides 1447–1467 on 12-LOX cDNA); antisense, 5′-GGC ACC ATG TCT GGC TGG CG-3′ (complementary to nucleotides 1831–1850). β-Actin primers were from Stratagene (La Jolla, CA, USA). PCR for 5-lipoxygenase (5-LOX; arachidonate:oxygen 5-oxidoreductase EC 1.13.11.34,) was performed according to [25] using 5-LOX cDNA (generous gift of J. F. Evans, Merck-Frosst, Pointe Claire-Dorval, Quebec, Canada) as positive control template. Nine microliters of PCR products were separated by electrophoresis on 1.2% agarose gels, stained by ethidium bromide, and photographed.

Measurement of 12-Lipoxygenase Activity

Adherent A431 cells were dislodged from the tissue flask with a cell scraper. After the cells were washed twice with PBS, they were homogenized in 25 mM Tris-HCl, pH 7.6, 1 mM EGTA containing aprotinin (5 μg/mL), leupeptin (10 μg/mL), and 1 mM phenylmethylsulfonyl fluoride. All samples were immediately used for 12-LOX activity assay or prepared for electrophoresis. Protein in each fraction was determined by the Bradford method [26] using BSA as a standard. For 12-LOX activity assay, samples were diluted in 50 mM Tris-HCl, pH 7.6 in a final volume of 100 μL at a protein concentration of 1–4 mg/mL. In activity assays studying the direct influence of compounds, inhibitors or their vehicle were preincubated with the cell homogenates at 37°C for 2 min. Reactions were initiated by adding arachidonate (10 μM) together with [^{3}H]arachidonate (1 μCi), incubated at 37°C for 20 min, and stopped with 200 μL acetonitrile/methanol/acetic acid (35:15:0.3) [27]. Following centrifugation, supernatants were analyzed for 12-[^{3}H]HETE by reverse phase-HPLC as reported [28].

Western Blot

After aliquots of the cellular homogenates were subjected to SDS-PAGE on 8% gels, proteins were electrophoretically transferred to nitrocellulose membranes and probed for 12-LOX with a 1:5000 final dilution of rabbit polyclonal antiserum to purified human platelet/HEL 12-LOX (Oxford Biomedical Research, Oxford, MI, USA) [27] as reported [29], using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) for detection. Quantitation of the 12-LOX band with internal standards was performed using a laser densitometer as reported [12, 30]. Under the conditions used, the intensity of the 12-LOX band was proportional to the amount of cellular homogenate loaded onto the gel. Detection of 15-lipoxygenase (15-LOX; arachidonate:oxygen 15-oxidoreductase, EC 1.13.11.33) was performed analogously using a 1:500 diluted polyclonal 15-LOX antiserum and purified 15-LOX protein (kind gifts from H. Kühn, Berlin, Germany) as positive control.

Inhibitors

DAPH1 was kindly provided by Dr. N. B. Lydon (Ciba-Geigy Ltd., Basel, Switzerland), PD 153035 by Dr. D. W. Fry (Parke-Davis, Ann Arbor, MI, USA), and BHPP by Biomide Corporation (Grosse Pointe Farms, MI, USA). All

compounds were dissolved in DMSO and were not toxic to the cells at the concentrations used as judged by trypan blue exclusion.

RESULTS AND DISCUSSION

Inhibition of EGF-R Tyrosine Kinase Results in Loss of 12-LOX Activity

Our study focused on whether functional EGF-R tyrosine kinase was necessary to maintain the constitutive, basal expression of 12-LOX in A431 cells, and whether arachidonate metabolites originating from EGF-activated 12-LOX played a linking role in the signal transduction pathway between EGF and 12-LOX expression. We first tested two inhibitors, PD 153035 and DAPH1, with high selectivity for the tyrosine kinase of EGF-R and of p185c-erbB2, a closely related member of the EGF-receptor family [22, 23]. Treatment of A431 cells for 24 hr with PD 153035 (0–10 μM) resulted in a dose-dependent decrease in 12-LOX activity (Fig. 1). A drop by 20% in cellular 12-LOX activity was observed with PD 153035 as low as 50 nM (Fig. 1); at this inhibitor concentration, EGF-R autophosphorylation in A431 cells is inhibited significantly [23]. 12-LOX activity dropped to 14% of control in cells treated for 24 hr with 10 μM PD 153035 (Fig. 1). These drug concentrations were tolerated by the cells without loss of viability during the experimental time period. The concentration of PD 153035 needed for half-maximal inhibition (IC_{50}) of cellular 12-LOX activity in A431 cells amounted to 0.3 μM (Fig. 1). This IC_{50} is higher than the reported IC_{50} (14 nM) of PD 153035 for EGF-R autophosphorylation [23]. This difference may be due to, for example, instability or metabolic inactivation of the inhibitor during our prolonged experimental time periods. However, even at μM concentration PD 153035 is a very specific inhibitor of EGF-dependent cellular processes, whereas PDGF- or bFGF-dependent mitogenesis or early gene expression are unaffected [23].

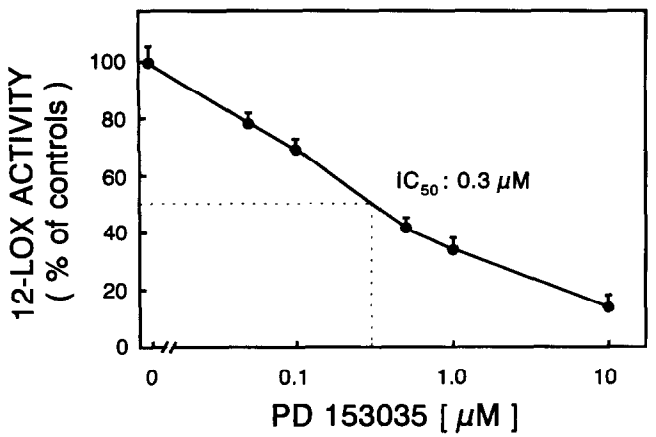

FIG. 1. Loss of cellular 12-LOX activity after treatment with PD 153035. A431 cells were cultured for 24 hr with DMSO (0.1%, v/v; vehicle control) or in the presence of PD 153035 at indicated concentrations. Cellular 12-LOX activity at that time point was determined as 12-HETE generation from arachidonate (see Materials and Methods). Data are mean values ± SD from three separate experiments each run in duplicate.

Another EGF-R tyrosine kinase inhibitor, DAPH1, also caused a decrease in cellular 12-LOX activity. Compared with vehicle-treated control cells, 12-LOX activity of cells cultured for 24 hr in the presence of 10 μM DAPH1 was only 39 ± 2% (mean ± SD, n = 5 experiments). This inhibitory efficacy correlates well with the reported IC_{50} (1–10 μM) of DAPH1 on EGF-R autophosphorylation [22]. Our data suggest that not only the EGF-inducible [9–11] and glucocorticoid-sensitive part of 12-LOX expression [31], but also the basal cellular level of 12-LOX activity are sensitive to tyrosine kinase inhibition of the EGF-R. This is in line with earlier findings that general tyrosine kinase inhibitors such as genistein and herbimycin A also suppress 12-LOX expression in A431 cells [12].

The observed decrease in 12-LOX activity after treating cells with EGF-R tyrosine kinase inhibitors could be the result of a direct inhibitory action of either PD 153035 or DAPH1 in the 12-LOX activity assay. To test this possibility, we performed 12-LOX activity assays in the presence of these compounds using homogenates from untreated A431 or HEL cells [32] as 12-LOX source. As a result, neither PD 153035 nor DAPH1 at concentrations of up to 100 μM significantly affected 12-LOX activity under these conditions: at 1, 10, or 100 μM of PD153035 (DAPH1), 12-LOX activity amounted to 108 ± 5 (104 ± 12)%, 105 ± 1 (82 ± 4)%, or 78 ± 3 (89 ± 8)% of controls, respectively (mean ± SD, n = 3 experiments). Thus, the observed loss of 12-LOX activity after 24 hr incubation of cells with either PD 153035 or DAPH1 cannot be ascribed to a direct interfering action of these compounds with the 12-LOX enzyme.

EGF-R Inhibitors Induce Decrease in 12-LOX Protein

The apparent loss of cellular 12-LOX activity as a result of EGF-R tyrosine kinase inhibitor action was paralleled by a reduced cellular content of 12-LOX protein under these conditions. As shown in Fig. 2, 12-LOX protein in A431 cells was diminished in a dose-dependent manner after a 24-hr treatment with PD 153035 (0–10 μM). This PD 153035-induced decrease in 12-LOX protein was detectable after treatment of cells with drug concentrations as low as 0.5 μM. Cellular content of 12-LOX protein dropped to 25% of control at 10 μM PD 153035, as judged from quantitation of Western blot data (Fig. 2, upper panel). Using DAPH1 (10 μM, 24 hr) as inhibitor of EGF-R tyrosine kinase, the amount of cellular 12-LOX protein was also diminished to 24% of controls. Although Western blotting gives at best semiquantitative data, these results support the above-mentioned findings regarding the observed loss of 12-LOX activity, which is evidently due to a reduced cellular amount of 12-LOX protein after treatment of A431 cells with EGF-R tyrosine kinase inhibitors.

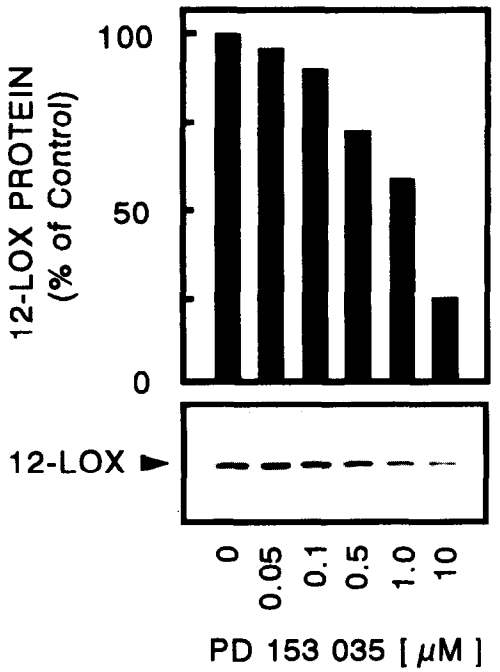

FIG. 2. Dose-dependent decrease in cellular 12-LOX protein induced by PD 153035. A431 cells were treated for 24 hr with DMSO (0.1%, v/v; vehicle control) or with PD 153035 at indicated concentrations. Cells were harvested and samples containing 15 μg of total cellular proteins each were subjected to Western blot for 12-LOX (Lower panel) with densitometric quantitation of 12-LOX bands (Upper panel; data represent mean values from two separate experiments).

The time course of suppression of 12-LOX protein expression by EGF-R tyrosine kinase inhibition is illustrated in Fig. 3. Upon treatment of A431 cells with 1 μM PD 153035 for varying time periods, 12-LOX protein and activity in these cells diminished continuously. In this set of experiments, the decrease in 12-LOX protein was even larger than that observed in the dose-dependency studies

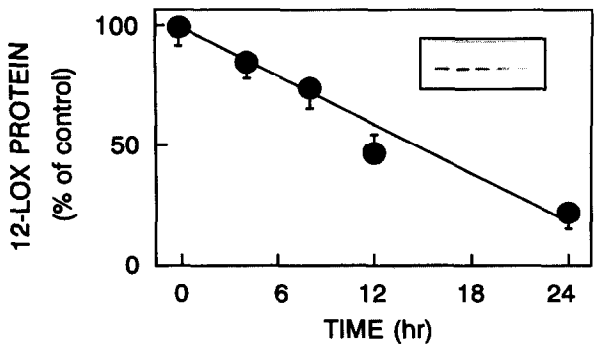

FIG. 3. Time course of PD 153035-induced decrease in 12-LOX Protein. A431 cells were treated for indicated periods of time with 1 μM PD 153035. Cell lysate samples (15 μg of protein each) were analyzed by Western blot and densitometry as in Fig. 2. Data are mean values ± SD from two separate experiments. Insert shows 12-LOX Western data with lanes representing (from left to right) samples after 0, 4, 8, 12, and 24 hr of PD 153035 treatment, respectively.

(Fig. 2), which may be due to the methodic limits of Western blot quantitation despite careful calibration [12]. At this inhibitor concentration sufficient for complete inhibition of EGF-R tyrosine kinase [23], 12-LOX protein was suppressed to 50% of untreated control level within approximately 12 hr (Fig. 3). The corresponding 12-LOX activity levels after 4, 8, 12, and 24 hr incubation with 1 μM PD 153035 amounted to 92 ± 2, 72 ± 2, 65 ± 7, and 37 ± 10% of controls, respectively (mean ± SD, $n = 4$).

Suppression of 12-LOX Protein Expression by Inhibition of 12-LOX Activity

Because EGF can elicit acute 12-LOX activity in the presence of sufficient free intracellular Ca^{2+} [12], we wondered whether EGF-dependent expression of 12-LOX may also depend on mediators derived from 12-LOX activity. To test this possibility, we first checked the efficacy of the LOX inhibitor BHPP in inhibiting 12-LOX activity of normal A431 cell homogenates. BHPP inhibited this 12-LOX activity with an IC_{50} of 0.45 μM (27%, 52%, 64%, or 86% inhibition at 0.1, 0.5, 1.0, or 5.0 μM, respectively), as compared to an IC_{50} of 0.1 μM in platelets [33]. In intact cultured A431 cells, addition of 10 μM BHPP 24 hr before testing the cellular 12-LOX activity resulted in a substantial loss of cellular 12-LOX protein (60% of vehicle-treated controls) and of 12-LOX activity (21% of controls). These results indicate that prolonged presence of BHPP not only prevents the enzymatic activity of 12-LOX, but also leads to a diminished cellular expression of 12-LOX protein. Several reasons support this interpretation of our data despite the fact that BHPP is not a selective inhibitor of 12-LOX (IC_{50} for 5-LOX–dependent leukotriene formation in RBL2H3 cells: 0.4 μM) (W. Hagmann, unpublished observation, 1994). First, the observed BHPP effect on 12-LOX expression is not likely due to inhibition of 5-LOX activity, because we could not detect 5-LOX in our A431 cells by reverse transcription-PCR [25] or via 5-HETE generation from arachidonate by reverse phase-HPLC [12]. Second, 15-LOX was not detectable in our A431 cells by Western blots, nor did we observe 15-HETE generation according to reverse phase-HPLC analysis [12]. Third, preliminary data indicate that culturing A431 cells in the presence of 0.1 μM 12(S)-HETE can elicit an increased 12-LOX expression, which further argues for a role of 12-LOX–dependent arachidonate metabolism in upregulating 12-LOX expression. Fourth, whereas we cannot exclude an effect by BHPP on cellular targets other than 12-LOX or downstream of 12-LOX in this 12-LOX–dependent signaling pathway, BHPP was reported to have no inhibitory activity on prostaglandin or 15-HETE production [21, 34].

Influence of EGF-R Tyrosine Kinase Inhibitors and of BHPP on 12-LOX mRNA Level

Our finding of a decrease in cellular 12-LOX protein due to EGF-R tyrosine kinase inhibition or to prevention of 12-

LOX activity can be the result of diminished transcription of the 12-LOX gene, of inhibited translation of its mRNA into protein, or of enhanced degradation of 12-LOX mRNA or protein. From our Western analyses, we cannot exclude 12-LOX protein degradation. On the other hand, the Western blots gave no direct indication for 12-LOX protein degradation under the influence of EGR-R tyrosine kinase inhibitors (Figs. 2 and 3), because residual 12-LOX with the correct molecular mass was detectable under all conditions, whereas none of its immunoreactive degradation products were [12]. In contrast, 12-LOX mRNA content in cells treated for 24 hr with 1 μM PD 153035, 10 μM DAPH1, or 10 μM BHPP dropped to very low levels or was undetectable as judged from reverse transcription-PCR (Fig. 4). Expression of cellular β-actin as a control was not affected by the inhibitors (Fig. 4). Taken together, our data indicate that inhibition of EGF-R tyrosine kinase or of 12-LOX activity results in a diminished presence of cellular 12-LOX mRNA in A431 cells, which accounts for the suppression of 12-LOX protein expression and loss of 12-LOX activity. This is in line with evidence reporting that omission of sufficient growth factors such as serum or EGF from culture medium can result in a decreased presence of 12-LOX in both cytosol and membranes of A431 cells [12]. Because we demonstrate in this study that both total cellular 12-LOX protein (which is mainly cytosolic) and 12-LOX activity (which is mainly in membranes) of A431 cells are decreased in parallel under the influence of EGF-R tyrosine kinase inhibitors, the observed drop in 12-LOX mRNA levels elicited by these inhibitors is not likely to affect preferentially either cytosolic or membrane fractions of 12-LOX but rather leads to a general loss of cellular 12-LOX.

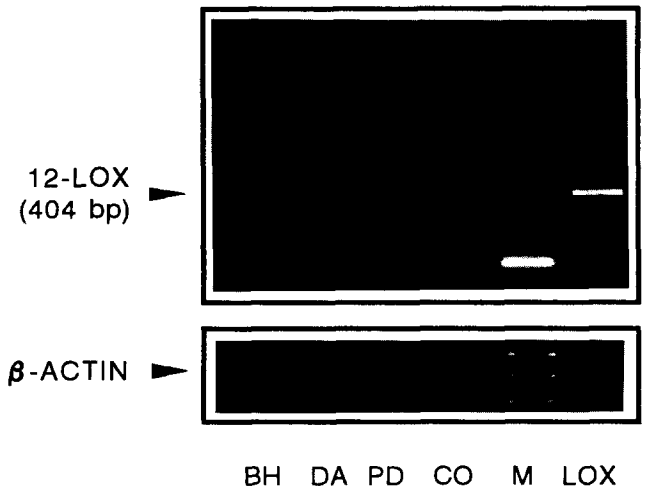

FIG. 4. Decrease in 12-LOX mRNA by PD153035, DAPH1, and BHPP. A431 cells were cultured for 24 hr in the presence of either vehicle (DMSO; Co), 1 μM PD 153035 (PD), 10 μM DAPH1 (DA) or 10 μM BHPP (BH). Total RNAs from these cells were reverse transcribed and amplified by PCR in the presence of primers specific for either platelet-type 12-LOX (upper panel) or β-actin (lower panel). Final PCR products were separated on a 1.2% agarose gel. LOX: PCR-amplified isolated 12-LOX cDNA (404 bp fragment). M: Size markers 123 bp ladder.

The mediators most likely responsible for 12-LOX-dependent expression of 12-LOX are 12(S)-hydroperoxyeicosatetraenoic acid (12(S)-HPETE) or 12(S)-HETE [10, 12] as major arachidonate metabolites generated via 12-LOX action in A431 cells, and preliminary data on 12-HETE–induced upregulation of 12-LOX protein levels support this notion. Further studies should clarify to what extent and at which target 12(S)-HPETE and/or 12(S)-HETE can directly or indirectly influence the expression of 12-LOX. So far, reported regulatory elements in the human 12-LOX gene include SP1, AP2, and NFκB, with the latter having been demonstrated to function as a negative transcriptional regulator in its NFκB/Rel heterodimer form [35]. However, the ligands for three regions exerting positive transcriptional regulation of the 12-LOX gene have not been identified yet [35].

In summary, our results shed new light on the established signal connection between EGF action, 12-LOX activity, and 12-LOX expression in EGF-responsive cells. In particular, we suggest in this article that (a) 12-LOX expression in A431 tumor cells depends to a major extent on functional EGF-R tyrosine kinase and that (b) 12-LOX activity in these cells positively contributes to the regulated expression and/or stability of their 12-LOX mRNA. On the other hand, the EGF-R and the closely related *c-erbB2* gene are frequently amplified or overexpressed in various human tumors such as mammary and ovarian carcinomas and adenocarcinomas [36, 37], and arachidonate metabolites generated via 12-LOX activity are known to enhance the metastatic potential of tumor cells [13]. Our demonstration of a possible functional link between EGF-R tyrosine kinase, 12-LOX activation, and 12-LOX-dependent expression of 12-LOX in A431 cells suggests that the combined use of inhibitors of EGF-R tyrosine kinase and of 12-LOX may offer a therapeutic potential against appropriate 12-LOX–containing or 12-HETE–responsive tumor cells.

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