Involvement of lipoxygenase in lysophosphatidic acid-stimulated hydrogen peroxide release in human HaCaT keratinocytes

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Although it is now recognized that low levels of reactive oxygen species (ROS) are required for the mitogenic response, mitogen-induced signalling pathways that regulate ROS generation in non-phagocytic cells remain largely uncharacterized. Using a real-time assay for measuring hydrogen peroxide (H_2O_2) formation, we analysed H_2O_2 release in human HaCaT keratinocytes in response to lysophosphatidic acid (LPA), a mitogen for keratinocytes. LPA rapidly increased H_2O_2 release in HaCaT cells. Unlike LPA-induced mitogen-activated protein (MAP) kinase activation, LPA-stimulated H_2O_2 release was independent of the tyrosine kinase activity of the epidermal growth factor (EGF) receptor. Calcium chelators, phospholipase A_2 inhibitors, and lipoxygenase inhibitors effectively blocked

LPA-stimulated H_2O_2 release, whereas cyclooxygenase inhibitors were without effect. Addition of 5-lipoxygenase products 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and leukotriene B_4 , but not 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene C_4 , restored LPA-stimulated H_2O_2 release in cells treated with the lipoxygenase inhibitors nordihydroguaiaretic acid and Zileuton. These results suggest that the lipoxygenase products 5-HPETE and leukotriene B_4 are required for LPA-stimulated H_2O_2 release in HaCaT cells.

Key words: arachidonic acid, calcium, H_2O_2 , leukotrienes, phospholipase A_2 .

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are produced in a variety of cells in response to growth factors and cytokines. For example, platelet-derived growth factor (PDGF) increases H_2O_2 production in vascular smooth muscle cells [1], and basic fibroblast growth factor and tumour necrosis factor α increase ROS levels in chondrocytes [2]. Similarly, epidermal growth factor (EGF) transiently induces H_2O_2 production in human A431 epidermoid carcinoma cells, mouse NIH3T3 fibroblasts, and human HaCaT keratinocytes [3–5]. Increased amounts of H_2O_2 have also been detected in HeLa cells treated with lysophosphatidic acid (LPA) [6]. In lymphoid cells, interleukin-1 β and interleukin-2 produce ROS that mediate NF- κ B activation [7,8].

Little is known about the growth factor-regulated ROS production system in non-phagocytic cells. Since growth factor-stimulated ROS generation is inhibited by the flavoprotein inhibitor diphenylene iodonium (DPI), it appears that non-phagocytic cells may use a flavonoid-containing enzyme to produce ROS after growth-factor stimulation [2,4]. On the other hand, interleukin-1 β -induced ROS production in B cells and interleukin-2-induced ROS production in T cells have been found to be inhibited by lipoxygenase inhibitors, but not by DPI [7,8]. In fibroblasts, expression of constitutive active Ras or Rac1 leads to increases in intracellular ROS levels, whereas expression of a dominant negative Rac1 blocks ROS production induced by growth factor and active Ras [4,9,10]. These observations suggest that, similar to the NADPH oxidase of neutrophils, Rac is a regulatory component of the growth factor-stimulated ROS production system in fibroblasts.

At least two lines of evidence have pointed to a critical role of ROS in mitogenic signalling. First, removal of H_2O_2 by catalase abrogates tyrosine phosphorylation induced by PDGF and EGF, activation of the p44 and p42 mitogen-activated protein (MAP) kinase (extracellular signal-regulated kinase 1 and 2, ERK1 and ERK2) induced by PDGF, and DNA synthesis induced by PDGF and activated Ras [1,3,9]. Similarly, the antioxidant *N*-acetylcysteine blocks activation of the MAP kinase pathway induced by PDGF and LPA [1,6] and *c-fos* expression induced by basic fibroblast growth factor [2], while the catalase inhibitor aminotriazole potentiates LPA-induced activation of the MAP kinase pathway [6]. Second, expression of a Rac1 mutant lacking the insert region necessary for ROS generation blocks Raf-1-mediated DNA synthesis [10], while expression of a dominant negative Rac1 that interferes with ROS generation inhibits Ras-induced DNA synthesis [9].

LPA is a phospholipid that induces an array of cellular responses by binding to seven transmembrane domain receptors, such as Edg2 and Edg4, which are coupled to the heterotrimeric G protein G_i , G_q , and $G_{12/13}$ [11–13]. In particular, LPA is a mitogen for keratinocytes [11]. It is well known that several signal transduction pathways are activated by LPA [11–13]. These include: MAP kinase activation, calcium mobilization and arachidonic acid release, activation of the small G protein Rho, and inhibition of adenylate cyclase. In a number of cell lines, LPA induces tyrosine phosphorylation of EGF receptor [14–17]. Pre-incubation of cells with specific inhibitors of the EGF receptor tyrosine kinase blocks LPA-induced EGF receptor tyrosine phosphorylation and MAP kinase activation [14,17]. Thus, EGF receptor kinase mediates a signalling pathway of LPA that activates MAP kinase.

Abbreviations used: ROS, reactive oxygen species; LPA, lysophosphatidic acid; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; LTC $_4$, leukotriene B_4 ; LTD $_4$, leukotriene C_4 ; NDGA, nordihydroguaiaretic acid; DPI, diphenylene iodonium; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks balanced salt solution; HRP, horseradish peroxidase; PLA $_2$, phospholipase A_2 ; 4-BPB, 4-bromophenylacetyl bromide.

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Figure 1 LPA stimulates H₂O₂ release in HaCaT cells

(a) Serum-starved cells (1×10^6) were incubated with luminol and HRP in HBSS and treated with various concentrations of LPA at time 0. Chemiluminescence was recorded immediately at 5-s intervals. For clarity, data from some time points are not shown in the graph. (b) Cells were treated with catalase (0–2000 U) 1 min prior to stimulation with LPA (5 μM) and chemiluminescence was measured. The data represent the mean and standard deviations of a triplicate experiment that has been repeated. For points without error bars, the standard deviations are within the symbols.

We report here that LPA rapidly stimulates H₂O₂ release in human HaCaT keratinocytes. In contrast with the MAP kinase pathway, LPA-stimulated H₂O₂ production in HaCaT cells is independent of the EGF receptor tyrosine kinase. Importantly, we find that 5-lipoxygenase products 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and leukotriene B₄ (LTB₄) are required for LPA-stimulated H₂O₂ release in HaCaT cells.

MATERIALS AND METHODS

Materials

LPA, Type II horseradish peroxidase (HRP, Sigma catalogue no. P-8250), luminol, 4-bromophenylacetyl bromide (4-BPB), thapsigargin, 5-HPETE, LTB₄, leukotriene C₄ (LTC₄) were from Sigma. EGF, cell culture medium and solutions were from Life

Technologies. Aristolochic acid, BAPTA-AM, ionomycin, nor-dihydroguaiaretic acid (NDGA), indomethacin, NS-398, and 5-hydroxyeicosatetraenoic acid (5-HETE) were from Biomol. [³H]Thymidine was from NEN. [³H]Arachidonic acid was from American Radiolabeled Chemicals. Anti-EGF receptor antibody was from UBI. Anti-phosphotyrosine antibody RC20 was from Transduction Laboratories. Anti-Active-MAPK antibody was from New England Biolabs. PD158780 [17–19] was generously provided by Alan Kraker (Parke-Davis Pharmaceutical Research). Zileuton [20,21] was provided by Abbott Laboratories. Anti-MAP kinase antibody TR12 raised against ERK2 was kindly provided by Michael Weber (University of Virginia).

Cell culture and H₂O₂ detection

HaCaT cells were grown in 60-mm plates in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS). Cells were serum-starved for 24 h in serum-free DMEM prior to use for experiments. H₂O₂ was determined by the luminol and HRP-dependent chemiluminescence assay [5]. Serum-starved cells were rinsed with PBS, trypsinized, suspended in Hanks balanced salt solution (HBSS), and were washed twice with HBSS. Cells (1×10^6) were incubated in a test tube (75 × 12 mm, Sarstedt no. 55.476) in 0.5 ml HBSS containing 0.2% fatty acid-free BSA, 100 μM luminol, and 0.5 unit of HRP at 37 °C for 10 min. After which, cells were placed in the luminometer (Lumat LB 9507) and LPA was added at time 0. Chemiluminescence was recorded immediately at 5-s intervals. Unless otherwise indicated, each experiment was performed in triplicate and repeated at least once.

For calibration of the chemiluminescence signal with the H₂O₂ concentration, the chemiluminescence signal was measured within 5 s of H₂O₂ addition. H₂O₂ concentration was determined by spectrophotometry at 240 nm using a molar extinction coefficient of 43.6 M⁻¹·cm⁻¹. Control experiments for the direct effect of each reagent on chemiluminescence were performed without the cells. Except NDGA and Zileuton, all reagents tested did not directly affect the chemiluminescence signal. For NDGA and Zileuton, the contribution of the direct effect of these agents on H₂O₂-generated chemiluminescence signal was measured with exogenous H₂O₂ in the drug-treated HaCaT cells in parallel with LPA stimulation experiments under identical conditions.

Arachidonic acid release

Confluent HaCaT cells (in triplicate) in 12-well plates were incubated with 0.25 μCi/ml [³H]arachidonic acid (specific radioactivity: 100 Ci/mmol) in DMEM/10% FCS for 20 h. Cells were washed three times with DMEM. Approx. 75% of the radioactivity was incorporated into HaCaT cells under these conditions. Cells were serum-starved in DMEM (1 ml/well) for 3 h before LPA stimulation (5 μM, 5 min). At the end of LPA stimulation, each medium (0.5 ml) was transferred to a microfuge tube. After centrifugation at 10000 g for 10 min, radioactivity in 0.1 ml of the cell-free medium was determined by liquid scintillation counting. Where phospholipase A₂ (PLA₂) inhibitors were tested, the inhibitors in various concentrations were added to the cells 15 min (4-BPB) or 30 min (aristolochic acid) before LPA stimulation.

Other assays

Tyrosine phosphorylation of EGF receptor was analysed by immunoblotting with anti-phosphotyrosine antibody RC20 after immunoprecipitation with anti-EGF receptor antibody LA22 as described [17]. ERK activation was analysed by immunoblotting

with anti-Active-MAPK antibody that reacts only with active ERK1 and ERK2 [17]. DNA synthesis was determined by the [^3H]thymidine incorporation assay essentially as described previously [17,22].

RESULTS

LPA stimulates H_2O_2 release in HaCaT cells

To determine whether LPA increases H_2O_2 release in HaCaT cells, serum-starved HaCaT cells were incubated with luminol and HRP. Low chemiluminescence was detected in the absence of LPA. Addition of LPA (0.1–10 μM) caused a rapid increase in the chemiluminescence signal that peaked at approx. 20–30 s after LPA stimulation and returned to the basal level in about 5 min (Figure 1a). No change in chemiluminescence was detected when BSA control was added to the reaction mixture. Figure 1a shows that the intensity of the chemiluminescence signal increased as the concentration of LPA increased and saturated at 5 μM LPA. Although the chemiluminescence signal varied between experiments as observed previously [5], it was quite consistent within the same experiment. In a standard assay using 1×10^6 cells, 5 μM LPA typically produced a maximal chemiluminescence signal between 4×10^6 rlu/s and 8×10^6 rlu/s. A similar magnitude of the chemiluminescence signal was generated by 300–500 nM H_2O_2 .

To determine if the chemiluminescence signal was generated from H_2O_2 , catalase was added to the reaction mixture. Figure 1b shows that catalase diminished the LPA-stimulated chemiluminescence signal in a concentration-dependent manner. Complete inhibition of the LPA-stimulated chemiluminescence was achieved with 2000 U of catalase. Thus, the chemiluminescence signal is a valid measure for H_2O_2 . Together, these results indicate that LPA can stimulate H_2O_2 release in HaCaT cells.

LPA-stimulated DNA synthesis is partially inhibited by catalase

Previous studies have demonstrated the requirement of ROS for the mitogenic response [1,7,10,18]. To examine whether H_2O_2

Figure 2 Inhibition of LPA-stimulated DNA synthesis by catalase

HaCaT cells (50% confluence) were serum-starved for 24 h, and stimulated with 5 μM LPA in the absence or presence of various concentrations of catalase as indicated. DNA synthesis was measured by the [^3H]thymidine incorporation method. The LPA-stimulated DNA synthesis activity was calculated by subtracting the basal activity (119295 ± 27622 cpm).

Figure 3 The EGF receptor tyrosine kinase inhibitor PD158780 does not affect LPA-stimulated H_2O_2 release

Serum-starved HaCaT cells were incubated in HBSS containing luminol, HRP and PD158780 (a, 100 nM; b, 50 nM) or DMSO (0.1%, solvent control) for 15 min at 37 $^{\circ}\text{C}$. H_2O_2 release was measured after stimulation of cells with 5 μM LPA (a) or 17 nM (100 ng/ml) EGF (b).

generated in LPA-stimulated cells has any physiological function, we measured the effect of catalase on LPA-stimulated DNA synthesis. LPA stimulated DNA synthesis of serum-starved HaCaT cells about 4.5-fold as measured by [^3H]thymidine incorporation (Figure 2). Addition of catalase to the cells decreased the LPA-stimulated DNA synthesis in a concentration-dependent manner. A maximal 66% inhibition of LPA-stimulated DNA synthesis was observed with 2000 U of catalase (Figure 2), a concentration of catalase that also completely inhibited LPA-stimulated H_2O_2 production (Figure 1b). This result suggests that H_2O_2 produced in LPA-stimulated HaCaT cells is involved in the mitogenic response to LPA.

LPA-induced H_2O_2 release is independent of EGF receptor tyrosine kinase

Recently, several laboratories demonstrated that EGF receptor tyrosine kinase mediates a LPA-signalling pathway leading to

Figure 4 Inhibition of LPA- and EGF-stimulated tyrosine phosphorylation of EGF receptor and ERK activation by PD158780

Serum-starved HaCaT cells in 100-mm plates were pretreated with PD158780 (50 nM, 15 min) or DMSO (0.1%, mock) and then stimulated for 3 min with LPA (20 μ M), EGF (0.5 ng/ml), or BSA. EGF receptor was immunoprecipitated from cell lysates and analysed by immunoblotting with the anti-phosphotyrosine antibody RC20 (a). After removing antibody RC20 from the filter, the filter was probed with an anti-EGF receptor antibody (b). Portions of cell lysates (10 μ g protein) were separated on SDS/polyacrylamide gel, transferred to nitrocellulose filters and examined by immunoblotting with an antibody that only reacts with active ERK1 and ERK2 (c) or with an antibody (TR12) that recognizes both active and inactive ERK2 (d).

ERK activation [14,15,17]. To assess whether EGF receptor tyrosine kinase also mediates LPA-stimulated H_2O_2 release, cells were treated with PD158780, a tyrosine kinase inhibitor specific for the EGF receptor [17–19], and LPA- or EGF-stimulated H_2O_2 release was measured.

As shown in Figure 3b, EGF stimulated H_2O_2 release in HaCaT cells. However, a high concentration (40–100 ng/ml) of EGF was required to stimulate H_2O_2 release in serum-starved HaCaT cells (Figure 3b, and data not shown). PD158780 (50 nM) effectively inhibited EGF-stimulated H_2O_2 release (Figure 3b), whereas it had no detectable effect on LPA-stimulated H_2O_2 release even at 100 nM (Figure 3a).

To further confirm that PD158780 was effective in inhibiting the EGF receptor tyrosine kinase, serum-starved HaCaT cells were pre-treated with or without PD158780 (50 nM), stimulated with LPA or EGF, and analysed for tyrosine phosphorylation of EGF receptor and ERK activation. Figure 4 shows that LPA induced tyrosine phosphorylation of EGF receptor and activation of ERK1 and ERK2 in HaCaT cells. PD158780 effectively inhibited LPA- and EGF-stimulated tyrosine phosphorylation of EGF receptor and activation of ERK1 and ERK2, confirming the activity of PD158780. Together, these experiments indicate that LPA-stimulated H_2O_2 release in HaCaT cells is independent of the EGF receptor tyrosine kinase.

Figure 5 Requirement of calcium for LPA-stimulated H_2O_2 release

(a) HaCaT cells were pretreated with EGTA (5 mM) for 5 min or with BAPTA-AM (40 μ M) for 20 min and H_2O_2 release in response to LPA (5 μ M) was measured. (b), (c) Cells were treated with ionomycin (2 μ M) or thapsigargin (1 μ M) at time 0, and H_2O_2 release was measured by chemiluminescence.

Role of calcium and PLA_2 in LPA-stimulated H_2O_2 release

A previous study indicated that calcium was required for H_2O_2 production in HaCaT cells [5]. It is well known that LPA induces calcium mobilization [23,24]. As shown in Figure 5, LPA-stimulated H_2O_2 release was inhibited when cells were incubated with the intracellular calcium-chelator BAPTA-AM. Removal of

Figure 6 Inhibition of LPA-stimulated [³H]arachidonic acid and H₂O₂ release by PLA₂ inhibitors

(a), (b) LPA-stimulated [³H]arachidonic acid release was determined as described in the Materials and methods. Each sample of cell free medium was collected 5 min after LPA (5 μM) stimulation. Preliminary experiments had shown that the LPA-stimulated [³H]arachidonic acid release reached plateau within 5 min. The data were from two triplicate experiments. (c), (d) The relative chemiluminescence intensity was calculated using the peak values of the chemiluminescence signal (at 30 s after LPA stimulation) after subtracting the basal chemiluminescence signal (at 0 s). Data in (c) were derived from three duplicate experiments, while data in (d) were from a triplicate and a duplicate experiment. BPB, 4-bromophenylacetyl bromide; ARA, aristolochic acid.

extracellular calcium with EGTA also diminished the LPA-stimulated H₂O₂ release. On the other hand, ionomycin (a calcium ionophore) and thapsigargin (an endoplasmic reticular Ca²⁺-ATPase inhibitor) stimulated H₂O₂ release in HaCaT cells as previously reported [5]. These results indicate a critical role for calcium in LPA-stimulated H₂O₂ generation.

A potential effector of calcium is PLA₂ [25]. PLA₂ cleaves phospholipids at the *sn*-2 position to produce arachidonic acid [26]. LPA is known to induce arachidonic acid release from membrane phospholipids [27]. Figures 6a and 6b show that PLA₂ inhibitors 4-BPB and aristolochic acid, respectively, inhibited LPA-stimulated [³H]arachidonic acid release from HaCaT cells with IC₅₀ similar to that reported previously for inhibition of PLA₂ activity in other cells (Table 1). To determine if PLA₂ activity is required for LPA-stimulated H₂O₂ release, LPA-stimulated H₂O₂ release was measured in HaCaT cells treated with various concentrations of 4-BPB or aristolochic acid. As shown in Figures 6c and 6d and summarized in Table 1, both of the PLA₂ inhibitors blocked LPA-stimulated H₂O₂ release in HaCaT cells in a concentration-dependent manner.

Lipoxygenase activity, but not cyclooxygenase activity, is required for H₂O₂ release in response to LPA

Arachidonic acid is metabolized either by the lipoxygenase pathway to 5-HPETE, 5-HETE and leukotrienes or by the

Table 1 Inhibition of LPA-stimulated [³H]arachidonic acid and H₂O₂ release in HaCaT cells by PLA₂ inhibitors

The IC₅₀ data for [³H]arachidonic acid (AA) and H₂O₂ release are derived from Figure 6. The reported IC₅₀ values are from Lanni and Becker [28] (4-bromophenylacetyl bromide), and Rosenthal et al. [29] (aristolochic acid).

PLA ₂ inhibitor	IC ₅₀ (μM)		
	Reported	AA release	H ₂ O ₂ release
4-Bromophenylacetyl bromide	10	12	9
Aristolochic acid	40	50	40

cyclooxygenase pathway to prostaglandins and thromboxanes. Incubation of HaCaT cells with the lipoxygenase inhibitor NDGA resulted in a concentration-dependent inhibition of LPA-stimulated H₂O₂ release (Figure 7a). The basal chemiluminescence signal was also reduced by NDGA (Figure 7a). This is partly due to the fact that NDGA can slightly affect chemiluminescence. In contrast, no inhibition of LPA-stimulated H₂O₂ release was observed in cells treated with the cyclooxygenase-1 and cyclooxygenase-2 inhibitor indomethacin and the cyclooxygenase-2 specific inhibitor NS-398 at the highest

Figure 7 Lipoygenase inhibitor but not cyclooxygenase inhibitors blocks LPA-stimulated H₂O₂ release

HaCaT cells were pretreated for 10 min with the indicated concentrations of NDGA, 28 μM indomethacin, 16 μM NS-398, or mock-treated with 0.1% ethanol, and then stimulated with 5 μM LPA at time 0. H₂O₂ release was measured by chemiluminescence. Control experiments showed that in cells treated with 1 μM NDGA, the direct effect of NDGA on peroxide-generated chemiluminescence contributed to 17% of the reduction in chemiluminescence intensity.

concentration tested (28 μM indomethacin, 16 μM NS-398, respectively, Figure 7b). These concentrations are ×28 the IC₅₀ of indomethacin and ×4 the IC₅₀ of NS-398 for inhibition of cyclooxygenases. These results suggest that one or more lipoygenase products, but not the cyclooxygenase products, are required for H₂O₂ generation in response to LPA in HaCaT cells.

If a lipoygenase product was required for LPA-stimulated H₂O₂ release, addition of this compound would be predicted to alleviate the inhibition by NDGA. HaCaT cells are known to express 5-lipoygenase [27], which generates 5-HPETE; 5-HPETE is then converted to 5-HETE, LTB₄, or LTC₄. Figure 8 shows that addition of exogenous 5-HPETE or LTB₄, but not 5-HETE or LTC₄, partially restored LPA-stimulated H₂O₂ release in NDGA-treated cells.

Figure 8 5-HPETE and LTB₄, but not LTC₄ and 5-HETE partially restore LPA-stimulated H₂O₂ release in cells pretreated with NDGA

HaCaT cells were pretreated with NDGA (ND; 2 μM) for 10 min at 37 °C in HBSS containing luminol and HRP. 5-HPETE (5HP; 0.86 μM), LTB₄ (B4; 0.86 μM), LTC₄ (C4; 4 μM), or 5-HETE (5HE; 2 μM) was added during the last 5 min of the 10 min incubation period. Following LPA (5 μM) stimulation, chemiluminescence was measured. The peak chemiluminescence signals at 20 s after LPA stimulation are shown.

Because NDGA has weak anti-oxidant activity, a well-characterized specific inhibitor of 5-lipoygenase, Zileuton [20,21], was used to further assess the role of 5-lipoygenase activity in LPA-stimulated H₂O₂ release in HaCaT cells. As shown in Figure 9, pre-incubation of cells with Zileuton resulted in inhibition of LPA-stimulated H₂O₂ release. This result agrees with the prediction that inhibition of 5-lipoygenase would block LPA-induced H₂O₂ release in HaCaT cells if 5-lipoygenase was involved in the LPA-induced H₂O₂ release in these cells. Importantly, addition of 5-HPETE or LTB₄ to Zileuton-treated cells restored most of the LPA-stimulated H₂O₂ release activity. Although 5-HPETE is a lipid hydroperoxide and may react with HRP, the chemiluminescence signal detected in the presence of 5-HPETE was inhibited by catalase, which only reacts with H₂O₂, indicating that the chemiluminescence signal was from H₂O₂ (data not shown). Also, we did not detect chemiluminescence when 5-HPETE was mixed with LPA, HRP and luminol in the absence of HaCaT cells.

DISCUSSION

We demonstrate here that LPA induces H₂O₂ release in HaCaT cells. The increase in H₂O₂ release is rapid (peaking at 20–30 s after stimulation) and transient. Our study differs from several previous studies [1–4,6,7,10] in that the extracellular H₂O₂,

Figure 9 Inhibition of LPA-stimulated H_2O_2 release by Zileuton and reversal of the inhibition by 5-HPETE and LTB_4

HaCaT cells were pretreated with Zileuton ($2 \mu\text{M}$) for 10 min at 37°C without or with the addition of 5-HPETE ($0.86 \mu\text{M}$) or LTB_4 ($0.86 \mu\text{M}$) during the last 5 min of pretreatment and then stimulated with LPA ($5 \mu\text{M}$). The basal chemiluminescence signals immediately before LPA stimulation and the peak chemiluminescence signals at 20 s after LPA stimulation are shown. The data are the average and standard deviations of two independent experiments performed in duplicate. Control experiments showed that the direct effect of Zileuton ($2 \mu\text{M}$) on peroxide-generated chemiluminescence contributed to 40% of the reduction in chemiluminescence intensity.

instead of the intracellular H_2O_2 , was measured. The chemiluminescence method used in this study offers a sensitive, consistent assay that can measure H_2O_2 generation in real time. This allows us to begin dissecting the signalling pathways leading to H_2O_2 generation in LPA-stimulated cells.

Although LPA may increase extracellular H_2O_2 level by inhibiting H_2O_2 degradation, several lines of evidence suggest that this is not the likely mechanism for the LPA-stimulated H_2O_2 release we observed in HaCaT cells. First, incubation of HaCaT with various concentrations of the catalase inhibitor aminotriazole did not lead to an increase in the chemiluminescence signal in our assay. Second, the LPA-stimulated increase in H_2O_2 release was transient. After the chemiluminescence signal returned to the basal level, a second addition of LPA to the cells had no effect on the chemiluminescence signal, whereas addition of sphingosine-1-phosphate (a phospholipid that binds to different G-protein-coupled receptors) evoked a second rise of the chemiluminescence signal (data not shown). Therefore, the LPA-stimulated H_2O_2 release was desensitized after the initial LPA treatment. This result indicates that LPA increases H_2O_2 release by activating a signalling pathway rather than by simply inhibiting H_2O_2 degradation.

It is unclear whether H_2O_2 is generated inside or outside of the plasma membrane in HaCaT cells and in other non-phagocytic cells in response to LPA and growth factors. H_2O_2 is a neutral molecule and is known to diffuse rapidly across the membrane [30]. Importantly, our study shows that catalase added to the culture medium inhibits LPA-stimulated DNA synthesis in HaCaT cells, suggesting that the H_2O_2 we detected is involved in the LPA-induced mitogenic response. Although we have not ruled out the scenario that extracellular catalase may be taken up by HaCaT cells to degrade intracellular H_2O_2 , a more likely possibility is that removal of extracellular H_2O_2 increases the

H_2O_2 gradient across the plasma membrane resulting in an indirect reduction of the intracellular H_2O_2 level. In addition, some of the H_2O_2 effectors involved in the mitogenic response may be located on the cell surface.

Although EGF receptor tyrosine kinase mediates LPA-stimulated activation of the MAP kinase pathway [14,15,17] and EGF induces H_2O_2 release in HaCaT cells, our data indicate that EGF receptor tyrosine kinase is not involved in LPA-stimulated H_2O_2 release in HaCaT cells. Because the EGF receptor tyrosine kinase inhibitor PD158780 inhibits ERK1/2 activation in LPA-stimulated HaCaT cells, the observation that PD158780 does not affect LPA-stimulated H_2O_2 release also suggests that ERK1/2 are not involved in LPA-stimulated H_2O_2 release. In addition, LPA-stimulated ERK activation, which peaks at 3.5–4 min (data not shown), considerably lags behind the peak of H_2O_2 release. Therefore, the kinetic data also suggest that ERK1/2 are unlikely to be involved in LPA-stimulated H_2O_2 release.

Even though LPA-stimulated H_2O_2 release is not mediated by EGF receptor, it is possible that LPA and EGF could activate common signalling components to regulate H_2O_2 release. Like LPA, EGF increases intracellular calcium concentration [5]. A previous study indicated that calcium was involved in EGF-stimulated H_2O_2 generation [5]. Incubation of HaCaT cells with NDGA or Zileuton also blocked EGF-stimulated H_2O_2 release (data not shown), indicating that lipoxygenase activity is also required for EGF-stimulated H_2O_2 release in HaCaT cells.

Our experiments demonstrate the requirement of 5-lipoxygenase activity for LPA-stimulated H_2O_2 release in HaCaT cells. During the preparation of this manuscript, Bonizzi et al. [7] reported that interleukin-1 β -induced ROS generation in lymphoid (Raji) cells also required 5-lipoxygenase activity. However, it is unclear which of the 5-lipoxygenase products is involved in ROS production in interleukin-1 β -stimulated lymphoid cells. In another report [8], it was found that inhibition of 5-lipoxygenase activity suppressed CD28-mediated ROS formation in T lymphocytes and that LTB_4 could slightly increase interleukin-2 production, implicating the involvement of LTB_4 in ROS generation in T lymphocytes. A previous study showed that the 5-lipoxygenase products 5-HETE, LTB_4 and LTC_4 (all of these are derived from 5-HPETE) could be detected in HaCaT cells [27]. In particular, LTB_4 and LTC_4 are produced in HaCaT cells in response to calcium ionophore [30]. Our experiments show that exogenous 5-HPETE and its metabolite LTB_4 can compensate for the requirement of 5-lipoxygenase activity for LPA-stimulated H_2O_2 release in HaCaT cells, while 5-HETE and LTC_4 are ineffective. These data establish for the first time that 5-HPETE and LTB_4 are the 5-lipoxygenase products required for H_2O_2 generation in HaCaT cells.

It remains to be determined how 5-HPETE and LTB_4 mediate H_2O_2 release in response to LPA. In the absence of LPA, exogenous 5-HPETE and LTB_4 only weakly increase the chemiluminescence signal when they are incubated with HaCaT cells, HRP and luminol (Figure 9). Two conclusions can be drawn from this observation. First, 5-HPETE and LTB_4 are unlikely to be the sources of H_2O_2 produced in LPA-stimulated HaCaT cells. If 5-HPETE or LTB_4 were the sources of chemiluminescence observed in LPA-stimulated HaCaT cells, high levels of chemiluminescence signal would be detected in the absence of LPA. Second, 5-HPETE or its metabolite LTB_4 can not be the sole mediator of LPA-stimulated H_2O_2 release in HaCaT cells. Other components also need to be activated by LPA for HaCaT cells to generate H_2O_2 . In fact, incubation of HaCaT cells with the flavoprotein inhibitor DPI also blocked H_2O_2 release in response to LPA (data not shown), suggesting that a flavonoid-containing

protein is also involved in LPA-stimulated H_2O_2 release. Thus, a multiple-component system analogous to the NADPH oxidase of neutrophils may be responsible for H_2O_2 release in LPA-stimulated HaCaT cells. Significantly, our study identifies 5-HPETE and/or its metabolite LTB_4 as a critical component of this H_2O_2 generation system.

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